LIVER INJURY

Transactions of the Eleventh Conference
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JOSIAH MACY, JR FOUNDATION CONFERENCE PROGRAM

As an introduction to these Transactions of the Eleventh Conference on Liver Injury I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program We are interested first of all in furthering knowledge about liver injury and to this end the participants were brought together to exchange ideas experiences data and methods In addition to this particular goal however there is a further and perhaps more fundamental aim which is shared by all our conference groups This is the promotion of meaningful communication between scientific disciplines

The problem of communication between disciplines we feel to be a very real and a very urgent one the most effective advancement of the whole of science being to a large extent dependent upon it Because of the accelerating rate at which new knowledge is accumulating and because discoveries in one field so often result from information gained in quite another channels must be established for the most relevant dissemination of this knowledge

The increasing realization that nature itself recognizes no boundaries makes it evident also that the continued isolation of the several branches of science is a serious obstacle to scientific progress Particularly is it so in medicine that the limited view through the lens of one discipline is no longer enough For example today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation At the other extreme medicine is certainly a social science and through mental health must be concerned with economic and social questions The answer then is not further fragmentation into increasingly isolated specialties disciplines and departments but the integration of science and scientific knowledge for the enrichment of all branches This integration we feel can be encouraged by providing opportunities for a multiprofessional approach to given topics

Although the fertility of the multidiscipline approach is recognized adequate provision is not made for it by our universities scientific societies and journals And perhaps the presence of other hindering factors must be admitted Partly semantic in nature they

may also to some degree be psychological Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these emotional barriers

Thus, our meetings are in contrast to the usual scientific gatherings They are not designed to present neat solutions to tidy problems but to elicit provocative discussion of the difficulties which are being encountered in research and practice For this reason, we ask that the presentations be relatively brief and that emphasis be placed on discussion as the heart of the meeting Our hope is that the participants will come prepared not to defend a single point of view but to take advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way that they would talk with their own colleagues in their own laboratories

We have, now, twelve groups functioning under the Conference Program on the following topics adrenal cortex, aging, cold injury, connective tissues, consciousness, cybernetics, infancy and childhood, liver injury, metabolic interrelations, nerve impulse, renal function, and shock and circulatory homeostasis When a new conference is organized, the chairman, in consultation with the Foundation, selects fifteen scientists to be the nucleus of the group, and every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting, but for the purpose of promoting full participation by all members and guests, attendance at any meeting is limited to twenty-five It is inevitable that in no topic can we possibly include more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group like this is that it is necessary to leave out so many people whom we would like to include

The transactions of these meetings are recorded and published This is done because the Foundation wishes to make current thinking in a field available to all those working in it, and because it believes that conveying to those in other fields who are concerned with science for example, government officials, administrators, etc, the essential nature of scientific research is also an important problem in communication Logic is a vital aspect of science, but equally essential is the intuitive or creative aspect Research is as creative as the painting of a portrait or the compos-

ing of a symphony Although logic is of course, necessary in order to rearrange, to test and to validate research thrives on creativity which has its source in unconscious nonrational processes Unfortunately, however, in the finished products which are presented to the world through research reports this integral part of scientific endeavor is shriveled by the cold white light of logic By preserving the informality of our conferences in the published transactions we hope to give a truer picture of what actually goes on in the minds of scientists and of the role which creativity plays

FRANK FREMONT SMITH M D,
Medical Director

INTRODUCTORY REMARKS

Best There are many faces new to the Liver Conference here today so that it may be a good idea to have individual self intro ductions with an explanatory word about particular interests For myself I shall say that the interest I have in the liver stems from almost everything I have ever done Surely the key place or one of the key places for the action of insulin is in the liver Choline acts on the liver Heparin was named after the liver by Howell and it can be liberated in many interesting conditions from the liver although all commercial heparin is made from the lung

Thats enough from me On to Dr Lagerstedt who is not to say Lagerstedt Histological Institute University of Lund Sweden

STEN LAGERSTEDT I studied chemistry and genetics for two and a half years and then I started to study medicine all at the University of Lund I have worked in laboratories in Stockholm and Copenhagen but the bulk of my work has been performed in Lund

My greatest interest in the Conference is to hear some thing about the liver from others and to try to tell what I know about it then I hope that we shall be able to correlate the results of research performed by different methods

CECIL J WATSON Even though it may lose me my membership in this organization I must confess that my principal interest is in the pyrrole pigments

W STANLEY HARTROFT My interest in the liver is very largely con fined to its normal and pathologic microscopy

SIDNEY C MADDEN I am much interested in abnormal protein metabolism and the liver appears to be centrally con cerned in protein metabolism

JESSE L BOLLMAN My interest in the liver is rather *in reverse* because most of my studies have been on the rest of the body after complete removal of the liver

EPHRAIM SHORR I am particularly interested in the liver as a con tributor through some of the factors which it produces to circulatory homeostasis

DeWITT STETTEN JR My studies have been concerned chiefly with rates of biological processes and deviations from normal

in these rates incident to disease or other disturbances The liver is only one of many sites where these things may be studied

VICTOR M SBOROV I am interested in the liver as the principal organ of metabolism, the organ system interrelationships of the liver, and their application to clinical medicine

SAMUEL SCHWARTZ *I am chiefly interested in the porphyrin com* pounds and in the synthesis of porphyrins, hemoglobin and similar heme-compounds, using C^{14} labeled materials to trace intermediates *Fortunately for my status here* this morning, another one of the compounds which is present in the liver, in addition to those mentioned by Dr Best, is porphyrin, occurring either in the free form or combined to form cytochrome, catalase, etc Therefore, there seems to be quite a field correlating this organ *with porphyrin metabolism*

ARTHUR W POLLISTER I am a cytologist For a large part of my career, until about ten years ago, I was interested mainly in the structure of cells, as nearly every other cytologist was *Then the field began to change into a physiologic* cytology Physiology rapidly got inside the cell, and it has penetrated inside the liver cell more effectively than into any other type Thus, when Dr Best invited me to be here, I was very glad to have a chance to exchange my limited amount of information with real specialists on this well-known cell

M HANS ELIAS I am a vertebrate animal a member of that species which happens to know what a vertebrate animal is, and a member of that species which is interested in finding out where we vertebrates come from *As far as the liver* is concerned, I am in an unfortunate spot, because the liver of vertebrates seems to come from nowhere In the most primitive vertebrates, the hag fishes (Myxini), the liver is suddenly there but in the invertebrates it seems *not to exist The only principle in biology* which really interests me deeply is evolution, the question of where do I come from The story of my ancestry begins with the cosmic fogs and leads to the liver

I was originally a zoologist Then I got on the job *training in comparative anatomy as Professor* of Histology at Middlesex Veterinary College My interest in the liver began when I produced a film for teaching purposes a

few years ago* I had to document every statement with a picture, and one is not allowed to cover up misinformation by means of words, as authors of textbooks and lecturers can afford to do And so because I had to tell my story in pictures I found a few things which I shall tell you this evening It is a rule that no teaching film should contain material not previously published, and, as a result, not only a film strip but also two papers† came out of that effort

HANS POPPER I received my medical education in Vienna and was very much influenced in all my work and especially in my thinking by my teacher Hans Eppinger, who focused my interest on the liver I am primarily interested in the morphologic changes in the normal and more so in the abnormal, liver, and the correlation of these structural changes with functional alterations as recognized by biochemical and other methods

HANS SMETANA Smetana of the University of Vienna Medical School Johns Hopkins Medical School Peking Union Medical College College of Physicians and Surgeons, Columbia University, Armed Forces Institute of Pathology I am interested in general pathology, and the liver happens to be one of the organs

JOHN L. TULLIS I have degrees from Yale University and Columbia's College of Physicians and Surgeons I interned at Bellevue Hospital and Presbyterian Hospital, where I was an assistant resident in gynecology and obstetrics in 1941, when the Navy called me to active duty They found in my record that I had had six months of pathology The Navy chooses one's career sometimes, and they considered my training in pathology would be most useful for their needs I received additional training under Charles Geschickter in Washington and Shields Warren in Boston and was certified in 1946 I am now on duty in the Naval Medical Research Institute in Bethesda

* ELIAS, H Discovery by illustration *Scient Monthly* 70 229 (1950)
——— Principles of scientific teaching film production with special reference to the medical film *J Am M Coll* 25 333 (1950)

† ——— A re-examination of the structure of the mammalian liver I Parenchymal architecture *Am J Anat* 84 311 (1949)
——— A re-examination of the structure of the mammalian liver II The hepatic lobule and its relation to the vascular and biliary systems *Am J Anat* 85 379 (1949)

I am interested in the liver chiefly because of the great importance of epidemic hepatitis as a military disease My chief interest recently has been research on the effects of ionizing radiations on tissues

MELVIN H KNISELY At no time since I have been grown has anyone asked me what I am interested in point blank Among this group of old friends I shall try to tell the truth

I was raised under a philosopher named Williams at Albion College Albion Michigan Among other things he defined a philosopher as a man who will seek out and face squarely all of the evidence all of the time Dr Williams introduced me to the *Autobiography of Benvenuto Cellini* which contains the statement that the best way to learn is to find a man who knows what you want to know and go study with him At the University of Chicago I was raised under Robert R Bensley Normand Hoerr and George W Bartelmez in anatomy In physiology I studied under Victor Johnson who is now at the Mayo Foundation and Ralph W Gerard and Ajax Carlson Later at the University of Copenhagen in Denmark I studied physiology under Dr Paul Brandt Rehberg who invented the first kidney clearance test and under Professor August Krogh

I think that my main interest is first of all problems of value how to tell what is important from what is less important how to make scales of importances The most important things I know are people and all importances *relate to the importance of ideas and things to people*

I would say my next interest is epistemology how to tell truth from error What are the tools which the human mind in all history has been able to devise to tell truth from error? That is the main thing if we can find out how to detect a mistake so that somehow with this brain inside of our heads we can have ideas which are accurate about things which are outside of our heads the problem of making or getting accurate ideas and of making descriptions pictures moving pictures and equations of reality

There is a very simple list of tools which men have used for telling truth from error and in every single instance where any man tries to tell truth from error he is never using more than a few out of a total list that is not longer than thirteen items

1 Instinct
2 Custom
3 Tradition
4 Consensus
5 Feeling
6 Sense experience
7 Intuition
8 Correspondence
9 Pragmatism
10 The Will to Believe
11 The Will Not to Believe
12 Authority
13 Coherence criterion

Curiously enough Brightman does not include the item of "Authority" as a test of truth even though from the observable behavior of human beings it would seem that reliance upon authority, or appeal to authority, is a test of truth currently used with great frequency

The coherence criterion is obviously the only test which one can trust even for short periods of time It is possible to say that one of the great values of mathematics is that it permits us to keep track of the numbers and kinds and magnitudes of specific observations in order to test the validity of the over all implications of concepts by a maximum application of the coherence criterion

I am deeply interested in learning to observe accurately and describe clearly This is the toughest thing I know how to see something for sure and describe it clearly and say no more than one means

And I am interested in the problem of devising experiments which clearly separate possible ideas We have several interpretations of a certain pattern of thought How can an experiment be devised which will clearly point out which ones are no good and which ones still have to be accepted because you cannot find out that they are no good? The only reason that we ever accept any idea is that we have not yet found out that it is no good (This is one application of the coherence criterion)

In terms of people I am deeply interested in how the living human body is constructed how it works what can go wrong with it and what we can do about it I dont care anything at all about any other living thing I will kill any number of frogs or mice or monkeys to find out anything I can use to educate myself so that I can look for things and know what to recognize and what to look for in people

FRANKLIN M HANGER I studied medicine at Johns Hopkins I am now in the Department of Medicine at Columbia University I am primarily a clinician and my interest in the liver lies chiefly in methods whereby hepatic disease may be recognized at an early and perhaps reversible stage, and also in studying factors that influence the outcome of liver injury, i e, those leading to irreversible damage and those promoting healing

ALLAN L GRAFFLIN I was trained at Johns Hopkins, under Dr E K Marshall, Jr I have served in the Department of Anatomy at Harvard, but am now back at Hopkins I have general interests in secretory processes wherever they occur, and have developed a particular interest in the processes as they occur under normal and abnormal conditions in the vertebrate liver

ROBERT KARK I am from Chicago, formerly of Capetown, South Africa, where I was influenced by Lancelot Hogben Then I was trained at Guy s Hospital primarily by Arthur Hurst and John Ryle later at the Thorndike in Boston by the late George Minot and by William B Castle I am a physician, and my main interest is in my patients, many of whom have damaged livers

A M RAPPAPORT I graduated in Prague, had surgical training in Berlin and was foreign assistant in surgery at the University of Paris For the last four years, I have been in the Department of Physiology of the University of Toronto I am mainly interested in circulatory aspects of liver physiology and liver pathology

JOHN WATERLOW Cambridge University, England, and Medical Research Council, London I don't work in any special place but where my work takes me, to West Africa and the West Indies My interest is human physiology, or perhaps I should say medical physiology, and particularly all the things that happen to people when they are undernourished, which I regard as one of the main clinical problems in [illegible] My [illegible] liver is just a part of that [illegible] bridge the gap between [illegible] science [illegible] medicine, and the liver [illegible] for t[illegible]

JOHN R NEEFE I am a [illegible] not to [illegible] strayed [illegible] re [illegible] been in [illegible] My [illegible] sity

of Pennsylvania Following that I have spent most of my time there with the exception of a too-short period with Dr Watson at Minnesota Most of my investigative dealings with the liver have had in one way or another something to do with hepatitis Most of the earlier work was in association with Dr Stokes

I have been rather interested in the multiple fields into which one may be directed by a single disease I found myself involved with an infectious disease with all the problems of epidemiology and preventive medicine sanitary engineering entomology pathology biochemistry metabolism physiology immunology even medico legal problems to say nothing of my dominant occupation in recent years that of the clinical problems of the diseased liver especially the carrier state in respect to hepatitis virus and the problem of early and perhaps as yet undefined liver injury in persons who think they are healthy (and may be)

CHARLES DAVIDSON First with the University of California and thence to Montreal for medicine and back to California in San Francisco for two years and finally in Boston for the last ten years at the Thorndike where I became interested following Dr Kark under Dr Minot in food and blood as Dr Minot used to call it We sort of fell into the liver because it was present at least as far as disease was concerned in such large quantities in the Boston City Hospital There is an old saying that I think expresses part of our interest in the liver It goes Is life worth living? It depends upon the liver"

JOSEPH STOKES I am a graduate of the University of Pennsylvania The first investigations that I carried out were with Dr George Minot and Dr Chester Jones in Boston Then I went back to the University of Pennsylvania working in the clinical field of pediatrics

I regard it as very fortunate that infectious diseases and metabolic diseases join in the liver and that some of us may be able to lessen our ignorance in these fields by such meetings as this

MOTTRAM TORRE I am a social psychiatrist interested in the conference process and problems of communication and profoundly ignorant in the field of the liver I am working in Washington with the State Department and the

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JOHN R NEEFE I am a native Pennsylvanian and seem not to have strayed very far from there since I have been in medicine My medical education was at the University

of Pennsylvania Following that, I have spent most of my time there, with the exception of a too short period with Dr Watson at Minnesota Most of my investigative dealings with the liver have had, in one way or another, something to do with hepatitis Most of the earlier work was in association with Dr Stokes

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Defense Department on the use of the conference as a communication process

PAUL GYÖRGY As a child, I liked to sneak into my mother's pantry, for obvious reasons Emil Fischer, the famous German chemist, called the liver "the pantry of the organism" Perhaps this is the psychoanalytic explanation of my present scientific interest in the liver

GEORGE S MIRICK I was graduated from Johns Hopkins and interned there Dr Longcope was then Professor of Medicine I went to Stanford for three years with Dr Bloomfield Following that, I was an assistant resident physician at the Rockefeller Institute Hospital in New York, where I worked with Dr Avery, later with Dr Horsfall In the Navy, I was a member of the Rockefeller Institute Research Unit and of NAMRU 2 under Dr Thomas Rivers, then back to Johns Hopkins, where I have been since

I am a clinician, particularly interested in infectious diseases, and, by constant exposure to liver problems, I have, I suppose, become most interested in infections of the liver

FREDERICK W HOFFBAUER I too am a clinician I was born in Minnesota and received my medical training at that State's University It has been my good fortune to be associated with Dr Watson's department for fifteen years

My interest in the liver came about by a rather direct approach viz by needle biopsy beneath the costal margin I find no sympathy for the views of that member of the House of Commons cited in Walter Cannon's book who, in speaking against the teaching of physiology in the London schools many years ago stated When the Author of the Universe hid the liver of man out of sight, He did not want frail human creatures to see how He had done it

My principal interest is in the pathogenesis of cirrhosis a subject about which I confess great ignorance

FRANK FREMONT SMITH Born in Florida, trained at the Harvard Medical School greatly influenced by my teacher Francis Peabody and by my first tutorial student Hans Stetten

After hearing a lecture by Lou Reed on the cerebro spinal fluid I became interested in that wonderful clear limpid liquid which I approached by the direct means of the needle Then I learned something which was very

important to me, and that was that I could not measure spinal fluid pressure accurately unless I took cognizance of the emotional state of the patient Curiously enough, the patients behaved in two different ways when they were anxious Some held their breath, which raised their venous pressure and increased the spinal fluid pressure, and then I would think they had a brain tumor when they didn't Others panted and lowered their venous pressure and their cerebrospinal fluid pressure, and then I didn't think they had a brain tumor when they did So I learned that one had to pay attention to emotions if the physiology of the organism was to be understood

From that, I became interested in human relations, communication, and in 1936 became what has been called a philanthropoid someone who acts as though he were a philanthropist but is spending other people's money

TABLE I

Relation of Respiratory Infections to Hepatitis Following Plasma Inoculation

Respiratory Infection		Hepatitis	
	No	No	Per cent
Present	202	93	46 0
Absent	548	171	31 2
Total	750	264	35 3

$p = 0\ 0001$

These data suggest the possible importance of some secondary, apparently unrelated and seemingly trivial, environmental factor in determining the susceptibility of human beings to this infection

Smetana Was the course of the disease in those with respiratory infection similar to that of the other group?

Mirick Yes, it was entirely similar As in most epidemics, there was great variation in the severity of infection and in the character of the infection from one individual to another, but considered as groups they were the same For instance, in this outbreak, about 24 per cent were nonicteric, but I think the diagnosis was fairly certain because Dr Shank and I were in a position to carry out a number of liver function tests and to examine the patients daily, and we included in our group only those who had typical symptoms and signs of the disease, and abnormalities in at least two of the three liver function tests which we used, which were the measurement of serum bilirubin thymol turbidity, and bromsulfalein retention

Kark Dr Mirick did all the 750 have liver function tests?

Mirick We did not do tests on all 750, only on patients who presented themselves with some symptoms of disease, plus about 100 more who denied symptoms but whom we knew to have been inoculated with the plasma There were 243 more with some symptoms whom we did not think had hepatitis * The patients, of course, did not know at any time what they had been inoculated with and we were careful not to let anyone know Therefore, the actual number studied was the number that had hepatitis (300) plus about 100 more that had received plasma but developed no

* Of these 243 patients 103 had received the vaccine 58 the control (plasma) and 82 had not been inoculated at this establishment

symptoms, plus 243 others who had some suggestive symptoms but in whom the diagnosis of hepatitis could not be established The patients I have listed here represent only those with symptoms of the disease who had abnormal function tests, and were considered to be sick enough to be hospitalized

Kark Plus about 200 more?

Mirick Probably about 200 more, yes who were not diagnosed as having hepatitis

Kark What about respiratory infection in the other 200?

Mirick The only ones whom I analyzed for respiratory infection were the 750 who had received the plasma inoculation

There was a third group of patients whom I have not mentioned who did not receive either vaccine or the control (plasma) inoculation Respiratory infection occurred equally, approximately, in the group that had received the influenza vaccine and in those who had received the control material In other words as far as testing the vaccine went, the experiment was a blank It exerted no effect on the subsequent development of respiratory disease in the group I don t have the exact data here, but they were considered to be equal by the people who conducted the experiment

Sborov Was there a significant difference in the incidence of the nonicteric form of hepatitis in the 27 so-called secondary cases as compared with the 273 who developed hepatitis following the plasma?

Mirick No clinically speaking the cases were identical There was an equal proportion of icteric and nonicteric cases in the two groups about one fifth, as I said of the total group was nonicteric

Smetana Were there any histologic differences in the two groups?

Mirick There were no histologic studies We did not do any biopsies and none of the patients died

It is of some interest that the group that we consider as contact cases had the same high incidence of allergic manifestations as the group that received the plasma inoculation

Sborov Were they all inoculated at the same time?

Mirick At the same time and they were inoculated by random selection In other words it was set up as an experiment There was no selection between the groups as to which got one material and which the other They each received their injection from a syringe and a needle that had been previously autoclaved I think there was no chance that it was transmitted in series down the line by the method of inoculation

Sborov Was the time of development of hepatitis in the vaccine group approximately the same as in the plasma group?

Mirick No it wasn t the same it tended to be later Those patients which we call secondary cases did not all receive vaccine Seventeen of them received no inoculation of any sort It can be seen in Figure 1 that these cases occurred toward the latter part of the epidemic

Hanger Would you return to your discussion of allergy? Was it an allergic reaction to the inocula?

Mirick I wish I knew I don t know what the patients were allergic to I used the term allergic merely because the symptoms and signs were those which we commonly associate with some form of allergy say serum sickness

Hanger At what time after the injection?

Mirick The majority of them developed the symptoms which I have called allergic on the same day that they developed other symptoms and signs more usually considered a part of the hepatitis picture that is gastrointestinal symptoms or jaundice However there were quite a few patients who developed the allergic symptoms up to 30 days before the first gastrointestinal symptoms and there were some others who developed them up to 15 days afterward I would say that in general the allergic symptoms were early symptoms of the disease

Kark Dr Hanger am I not correct in thinking that the older clinicians in their accounts of catarrhal jaundice described hives as a prodromal symptom of the disease?

Hanger Yes I think that will come up later Some of the manifestations of a viral infection are assumed to be allergic reactions to the antigen

Stokes We see a great many cases with what appear to be allergic manifestations We have now studied more than 1400 volunteers who have received both the serum hepatitis and epidemic hepatitis viruses and many of them have the allergic symptoms you have discussed Dr Neefe and I had a very ill volunteer a conscientious objector who early in the course of his viral hepatitis had a severe scarlatiniform rash We frequently see urticaria pain in the joints and other manifestations that appear to be allergic in nature

Fremont Smith As first symptoms?

Stokes They are more apt to come early

Sborov Are the allergic manifestations more frequent in cases of epidemic hepatitis?

Stokes I believe they are more frequent in epidemic hepatitis than in serum hepatitis, but I cannot give exact data on incidence

Mirick I realize that this is not, of course, an original observation about the allergic symptoms I think the incidence is rather high in this outbreak An epidemic described by Hawley, *et al*, (1) in England, following the use of mumps convalescence serum had a rather high incidence, about 40 per cent And certainly in our experience at the Rockefeller Institute, in the series that Dr Hoagland reported (2), about 3 per cent of the naturally occurring hepatitis cases had hives at least, and 12 per cent of them arthralgia, too

An analysis of those symptoms which occurred in the Cleveland patients who received the injection is this arthralgia alone in 32 of the patients hives alone in 30, pruritus in 18 rash in 15, and angioneurotic edema in 3 Those are the single symptoms Both arthralgia and pruritus occurred in 18 arthralgia and hives in 15, arthralgia and rash in 8, hives and rash in 5, the hives and angioneurotic edema combination in 4 and arthralgia and angioneurotic edema in 3 Three of those symptoms occurred in 3, arthralgia, hives rash, and angioneurotic edema in 1 That brings the total up to approximately 55 per cent of the entire number

Stokes Did the cases that had allergic manifestations show more respiratory difficulty?

Mirick I have not made that correlation yet I don't believe that there is any

Stokes I believe that is a very important point So often respiratory symptoms may be allergic in nature

Mirick Yes, that is a good point I will consider that

Neefe Were there any blood counts done on the group during that particular period?

Mirick Very few, not enough to talk about We occasionally did some white blood cell counts when the diagnosis was in doubt, but in the majority of the cases we relied merely on clinical observation plus the three liver function tests that I have mentioned

Neefe As to eosinophilia there are not too many data on counts during that particular stage

Mirick Yes I wish I had that information We were rather short handed so we did not get everything done that should have been I am sure We have no eosinophil counts

Sborov In that connection Dr Neefe Zimmerman (3) studied about 300 cases of clinical hepatitis in the European theatre, and reported a high incidence of eosinophilia early in hepatitis His

cases were not in the prodromal period and it was rather surprising to me to see the high incidence of eosinophilia he found in this one group

Neefe I ask because I saw not long ago a patient a close personal friend who acquired hepatitis He presented allergic phenomena associated with 17 per cent eosinophilia and a high white count a rather unusual peripheral blood picture for hepatitis in fact it made me doubt the diagnosis But there is little information available concerning counts in the first few days of the disease and I doubt that we are well informed concerning the possible variants

Tullis The microscopic picture of this disease often shows an infiltration of the eosinophils doesn t it Dr Smetana?

Smetana Yes it does but we found a generalized eosinophilia in only a very low percentage in our group of cases I believe Dr Sborov has data concerning this point

Sborov Unfortunately we have not consistently done white blood counts on our patients with hepatitis but Dr Zimmerman studied his cases very carefully and found a relatively high incidence

Neefe Certainly in the volunteers Dr Stokes we had a fair number of early counts It is not a part of the usual blood picture I am sure but we do not have observations on a large number who have had the other allergic phenomena They may differ in their hematologic reaction to hepatitis

Best Dr Mirick will you go on?

Mirick Ten years ago Fox Manso Penna and Parra (4) studied an outbreak of jaundice following the administration of three icterogenic lots of yellow fever vaccine in Brazil They pointed out that the incidence of hepatitis within villages and households did not conform to that which would be expected if governed only by chance They discussed like Cullinan (5) Findlay and MacCallum (6) and Theiler (7) before them the apparent double etiology of postvaccination icterus The nature of the second factor was considered to be (*a*) possibly an infectious agent (*b*) a chronic intoxication or (*c*) a state of lowered hepatic resistance due to alimentary deficiencies

MacCallum and Miles (8) failed to produce hepatitis in laboratory animals following exposure to X rays splenectomy injection of various arsenicals injections of alloxan and infections with certain bacteria However they reported that the inoculation of blood and feces from patients with infective hepatitis into rats on

a protein deficient diet, followed by blind passages of the tissues of these rats into other protein deficient rats, led to the production of necrosis of the liver and hemorrhages into various other organs The diet employed was deficient in protein (yeast 16 per cent, casein 4 per cent) and caused microscopic liver lesions in 9 per cent of the normal rats fed it for 60 days Unfortunately, these workers lost their agent after 9 passages and failed in subsequent attempts to reproduce their original results Dr Ratnoff and I have, likewise, failed to reproduce the experiment, although we didn't start with the same material as they did, we did use the same method as closely as we could We had a different strain of rat, and also the yeast was not identical It was probably impossible to reproduce exactly their conditions We used the blood and feces of patients with acute infectious hepatitis, as did MacCallum and Miles, but from different sources

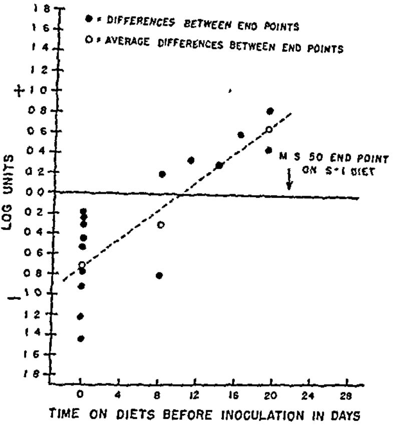
Neefe Was it known to be infectious?

Mirick The material we used was not known to be infectious At that time, we had no access to volunteers The material we used had not been tested but it was presumably infectious

I have been impressed by the possible importance of a second factor in determining susceptibility to hepatitis and for a number of years have attempted to apply this principle to the adaptation of the virus to some laboratory animal Some of the work has involved the study of environmental effects on the infection of mice with pneumonia virus of mice (PVM) This is a strictly pneumotropic virus causing overt infection only in mice, unless adapted to another species It can be titered reproducibly by measuring the 50 per cent maximum lesion score (MS 50) or by determining the hemagglutination titer of heated virus extracts Dr Leftwich and I (9 10) studied the effect of certain diets on the susceptibility of mice to this infection We showed that protein deficiency had no effect However, acute pyridoxine deficiency resulted in increased resistance to infection Chronic pyridoxine deficiency had the reverse effect and resulted in greater susceptibility of mice to the infection as shown in Figure 2

Kark Were you using paired feedings? Was the caloric intake of the animals the same?

Mirick The caloric intake was approximately the same They were all given as much to eat as they cared to have Each titration represents a total of 25 mice, and there were 5 in a jar, that is 5 inoculated with each dilution of virus, so that they were not compared mouse with mouse but group with group



II Influence of pyridoxine administered in the period before as well as after inoculation of virus J Exper Med 89 175 (1949)

Best That would not be paired feeding would it? They would not have the same caloric intake

Minck I think they would on the average Why do you think not?

Best If they are deficient in almost anything they won't eat as much

Minck The weight curves were followed for a period of 6 weeks The curve was the same at 5 weeks for the animals on the pyridoxine deficient diet as that for the animals on the complete diet At that time they began to lag in weight, but since these experiments were all completed in much less than 5 weeks it is unlikely that they were influenced

Best I dont know the latent period — Dr Gyorgy will probably know — but after a certain period their caloric intake goes way down

Gyorgy Maybe after 4 weeks Were these old mice?

Mirick No

Gyorgy The weight?

Mirick At the time of inoculation 10 Gm

Gyorgy Not longer than 4 weeks I am sure, if the diet was really free of pyridoxine

Mirick I think it was free We used Cowgills formula and we used as far as possible chemically pure ingredients to put into it I do not feel that the difference noted depended on lesser caloric intake Certainly, the mice on the protein deficient diet did lag markedly in weight The ones on 4 per cent protein never gained at all and yet that did not seem to bring out the difference in susceptibility that I have shown here with pyridoxine

Table II shows the titer of virus in the mouse s lung at 6 days This is 6 days after inoculation in two experiments comparing those on the pyridoxine free diet and those on the diet containing pyridoxine This virus was measured in two ways its infectivity titer in other mice and its capacity to agglutinate mouse erythro cytes It will be seen that in both the first and second experiments there was less virus in the animals acutely deficient in pyridoxine on the sixth day than in the animals who received it in their diet We did not measure virus in the chronically deficient animals which showed the increased MS 50 titer

The effect of cortisone on infection with PVM was tested (11) It was found that the administration of cortisone enhanced the severity of infection and the multiplication of the virus These findings were in agreement with those of others who have observed enhancement by cortisone of experimental infections with influenza poliomyelitis vaccinia Coxsackie mumps West Nile Ilheus and Bunyamwera viruses It is with interest that I note a recent report that Drs Melcher Blunt and Ragan (12) have produced a trans missible diffuse focal hepatitis in cortisone treated rats inoculated with icterogenic human serum

Finally the effects of urethane on PVM infection were tested (13) Urethane first seemed of interest because of its oncogenic activity but subsequent study has shown that many of its pharmacologic effects resemble those of the adrenal cortical steroids For example urethane may produce lympholysis eosinopenia suppression of inflammatory reaction and fibroblast growth chloride retention

TABLE II

Influence of Pyridoxine in Diet on PVM Content of Mouse Lungs

VIRUS SOURCE		AMOUNT OF VIRUS	
Lung Suspensions From Mice* On Various Diets After Inoculation		Infectivity Titer MS 50 End Point (Reciprocal of Log)	Hemagglutination Titer vs Mouse RBC (Final Dilution of Heated Lung Suspension)
Experiment No	Pyridoxine in Diet		
1 A	+	3 31	1 640
1 B	0	2.46	1 80
2 A	+	4 25	1 320
2 B	0	3 93	1·160

* *The mice were sacrificed on the 6th day after inoculation*

Reprinted by permission, from Leftwich, W B, and Minck, C S The effect of diet on the susceptibility of the mouse to pneumonia virus of mice (PVM) I Influence of pyridoxine in the period after the inoculation of virus *J Exper Med 89, 155 (1949)*

and loss of potassium and calcium, alkalosis, hyperglycemia, and increased gastric secretion We found that mice given urethane showed increased susceptibility to infection with PVM, and again the titers of virus obtained from their lungs were greater than from controls

This finding led Dr Janet Jordan and myself to the use of urethane as an adjuvant in attempting to adapt hepatitis virus to mice (14) Negative results had been previously obtained testing different diets and also several different adjuvants, lactophenin, carbon tetrachloride, and the hepatotoxic alkaloid monocrotaline, in rats mice or monkeys

Two sources of virus were tested with urethane One was pooled serum taken during the incubation period from patients who had been inadvertently inoculated with icterogenic sera The other was a 10 per cent saline extract of the liver of a patient (C B) who died of acute yellow atrophy 27 days after accidental inoculation This patient was a pathologist who cut his finger while autopsying a case of acute liver atrophy

These materials had been kept with dry ice until used. Neither source was tested for human infectivity. Weekly, semimonthly, and monthly serial passages were carried out using 0.3 ml. per mouse of a 10 to 15 per cent saline extract of pooled livers, spleens, and kidneys inoculated intraperitoneally. The mice inoculated with each source of material were given 0.1 per cent urethane to drink throughout the entire period. Two uninoculated control series, with and without urethane, were included. We have not observed any toxicity to the liver with this dosage of urethane alone.

It will be seen in Figure 3 that in the series of mice which were inoculated with C.B. liver, which was passed at four week intervals, and which received urethane, that in the fourth passage a disease appeared, and was subsequently maintained by serial passage through a total of 17 passages now. Urethane was not used after the fifth passage, since the disease was found to be transmissible in mice without that adjuvant.

It will also be seen that two mice of the other (GL) series also became somewhat sick. They did not develop ascites, but were thought to have some evidence of hepatitis. This also occurred in

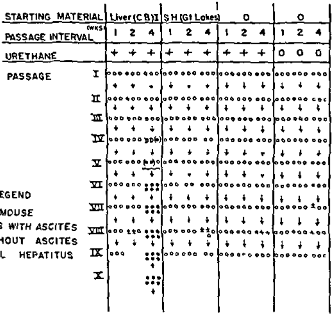


FIGURE 3. Attempts to isolate the virus of human hepatitis in mice.

several instances in the control series I shall come back to this observation later

Best Dr Mirick, I know nothing about this, but we see ascites in experimental animals with cirrhosis Can there be a real blockage with just acute inflammation? It is a clinical question

Mirick I think it is an unusual thing in man to see ascites with the acute disease I should like the other clinicians to comment on that There may be small amounts, but certainly massive amounts of ascites are not commonly seen in patients with acute hepatitis

Watson Well, we do see it every now and then I think we have to recognize clearly that ascites does occur with acute hepatitis It is rare, but it has been clearly recognized I have seen several cases where there was no other explanation where it cleared up and the patient recovered

Best Would you take that as the same mechanism as with cirrhosis or some change of permeability in the vessels?

Watson I really don't know I think there is probably more emphasis on other factors than the importance of obstruction perhaps some factors that Dr Shorr would be interested in assaying I don t know

Popper In approximately 30 per cent of cases of acute fatal hepatitis, the so-called acute yellow atrophy, considerable ascites may be noted A similar incidence has been reported by Lucke (15) He recorded ascites in approximately two thirds of his cases of actual fatal epidemic hepatitis, and Lucke and Mallory (16) noted it in one fourth of the fulminant cases of fatal epidemic hepatitis

Best Do you know anything about the mechanism?

Popper No sir

Mirick I don't know anything about the mechanism of ascites in this disease either, but it was a prominent feature in some of the mice Grossly the mice showed from a trace to 20 ml of yellowish ascitic fluid which clots on standing, has a cell count of about 1000 per cu mm, and contains about 3 per cent of protein The spleen is enlarged four times The liver may be enlarged mottled thickened and friable

Hartroft Were there eosinophils present in the ascitic fluid?

Mirick No there were no eosinophils in the ascitic fluid We checked that

Shorr Did you partition the protein Dr Mirick?

Mirick No we did not do that I don't know what the protein is I am sure there is some fibrinogen there because, as I say, it clots but I don t know what the rest of it is

Smetana Dr Mirick, when you say hepatitis, does that mean clinical hepatitis?

Mirick Well, I will show you sections from the livers It means inflammation in the liver, and the mice appear sick Yes, I suppose it means clinical hepatitis

Watson What percentage have jaundice?

Mirick Very few I have only seen three out of several thousand that had jaundice Microscopically, the livers show mononuclear cell infiltration around the portal and central veins, in the sub capsular area and in small scattered foci Occasional giant cells are seen The sinusoids are dilated, and frequently hemorrhages may be seen I believe that in Figure 4 you can see most of the features I have mentioned No inclusion bodies have been demon strated

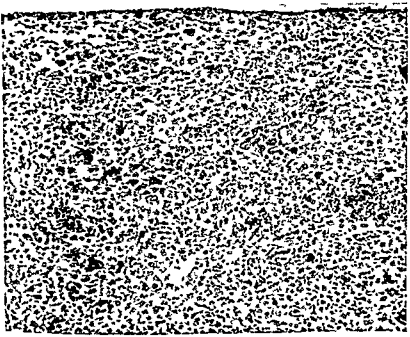


FIGURE 4 Photomicrograph (x150) of the liver of a mouse sacrificed 32 days after infection in the 7th passage No urethane was given Note diffuse infiltration par ticularly in the subcapsular area occasional giant cells dilatation of the sinusoids and disorganization of the hepatic architecture Reprinted by permission from Jordan J and Mirick G S Hepatitis in mice of presumed viral origin *Bull Johns Hopkins Hosp* 89, 327 (1951)

Best Dr Mirick you are going entirely on the weight of your animals, one group of mice might be gaining weight normally and the other might be developing ascites You would not know from weight alone

Mirick That is perfectly true When the mice develop much ascites there is little difficulty in recognizing it We have found that you can candle a mouse very nicely, like an egg If you take them into a dark room and transilluminate them you can see the ascitic fluid If there is any question and we want to keep the mouse alive, we do a paracentesis

Popper Is it now in order to ask some questions about histologic changes?

Best Yes, please

Popper First, what lesions do mice treated with urethane alone show?

Mirick They show normal livers indistinguishable from the mice that received water, that is with this dosage of urethane and for this length of time I am not sure what they would show if they received much more and for a longer time

Popper Higher doses of urethane produce characteristic histologic lesions Central necrosis and marked edema have been described by Doljanski and Rosin (17) in rats intoxicated with urethane, specifically, vascular changes with marked exudation The cellular inflammation however, I have not seen in urethane intoxicated rats I have never seen livers of mice intoxicated with urethane

Mirick Figure 4 was not from a mouse that received urethane it was obtained from an animal of a later passage The urethane came into the show only during the period of adaptation or what we thought was adaptation of the agent and after the fifth passage no urethane was given to these animals

Popper Did the first passages show the same histologic picture?

Mirick They showed no evidence of disease and no sections were taken We did not find any deviation from normal until the fourth passage in the series Urethane controls seemed normal at the seventh passage

Popper They showed thus a normal histologic picture

Mirick Yes

Best I am glad to have that cleared up too, because I was thinking of the possibility of damage from urethane

Popper I should like to ask one more question From what we know so far, a peculiar necrosis of the liver cells seems to be fairly characteristic for human virus infections of the liver This is a diffuse acidophilic or eosinophilic necrosis of the cytoplasm of the parenchymal cells Their nuclei become pycnotic and the cells finally leave the liver cell plates as if they were expelled from them Such cells are most characteristic in yellow fever, where they are designated as Councilman bodies Another variety is seen, as Dr Mallory has emphasized (18), in human viral hepatitis It is also seen in the virus hepatitis of dogs The few specimens I have studied were crowded with this type of eosinophilic necrosis some what similar to the Councilman bodies Have you seen any eosino philic necrosis of the liver cells in your mice?

Mirick If I have seen it, I have not recognized it I am not even entirely sure what it would look like You mean there are eosino phils in the liver?

Popper No, I was talking of liver cells with diffusely eosinophilic cytoplasm I am referring to isolated cells expelled from the liver cell plates and located in the tissue spaces

Mirick No, I have not seen that Let me say I may have seen it but not recognized it

Smetana I should like to ask whether these changes were limited to the liver

Mirick Not entirely We have a few sections of kidneys — I did not bring the slides along with me — which show some mononuclear infiltration That is the only other organ that we have recognized any changes in except for the spleen * The spleen shows what I think is the finding of a spleen in any infection in a mouse At least, I can't recognize it to be characteristic

Smetana The microscopic picture which you showed is unlike that seen in the nonfatal type of hepatitis in human beings There we consider the necrosis of individual liver cells, not of plates or cords the most important change The mononuclear reaction is not a primary but a secondary reaction to this injury of individual liver cells The picture showed necrosis of large groups of liver cells It is a lesion, but it is unlike that seen in the acute nonfatal type of hepatitis in human beings

Mirick That is certainly true I did not show these to try to say that they are like the human disease but merely to show what we do see Certainly, clinically and pathologically there are many deviations in what one sees

* Changes have also been seen in the pancreas

Smetana I believe the important point is that your diagnosis is based on histopathology

Mirick It is based on several things that we have looked at on the presence or absence of ascites, the gross appearance of the liver, and, if there is any doubt in our minds, on the histology We don't do sections on every liver that we see, because some of them are obviously diseased

Lagerstedt Have you made any studies with special staining reactions, for example, with the Feulgen stain, or studies of the basophilia, or have you investigated the composition of the nuclear or cytoplasmic proteins in these liver cells?

Mirick No, we have not done that yet Those are certainly things to be done The only things we have done, other than hematoxin and eosin, are stains for inclusion bodies

Lagerstedt There has been some interesting work done on virus infections with some of these methods I should think it would be of interest in connection with this, to investigate those things

Kniseley Have you sections of brains from these animals?

Mirick Yes we made sections many times We have tried to exclude encephalitozoon infection, which is best diagnosed by sections of the brain We have noticed no abnormalities in the brain

Hanger The heart muscle was all right?

Mirick Yes, the heart muscle seemed to be all right

Gyorgy May I ask you again Dr Mirick, during the first four transmissions, there was absolutely no pathology?

Mirick That's right

Gyorgy No ascites?

Mirick That's right

To continue with later observations ascitic fluid, blood and liver, spleen or kidney extracts separately have all been infectious, but only to a titer of 1 1000 All strains of mice at all ages tested are susceptible, but unpredictable negative experiments often occur, and mild forms of the disease are difficult to recognize All routes of injection including intracerebral have resulted in liver disease, but inoculation intranasally and per os have failed Guinea pigs, rabbits hamsters, monkeys and embryonated eggs were not obviously infected Infectivity for mice survives storage for at least 3 hours at room temperature 6 weeks at 4 degrees C, and 6 months at −70 degrees C It is usually lost by lyophilization or heating at 56 degrees for one hour or more Actually, about half of the time

under that degree of heating the infectivity is lost, and about half the time it survives

We have been unable to filter the agent through a Seitz pad, nor to demonstrate infectivity of the supernatant after centrifugation at 3000 r p m for one hour

These observations do not necessarily imply a large particle size to an agent of this low infectivity titer

Extensive efforts to identify the cause of this disease are being made Bacteriological cultures, including those for pleuropneumonia like organisms, have been negative No leptospira were seen Moreover, Dr Beeson found no agglutinins against eight strains of leptospira in the convalescent sera from our mice

Encephalitozoon infection may produce in mice a picture similar to that which we have described (19) However, none of these parasites have been found in our mice and rabbits do not become infected That was the main purpose of making brain sections, because there one sees the encephalitozoon parasite if it is to be found

The disease bears only a superficial resemblance to Ehrlich s ascitic tumor (20) The latter differs in the variability of its incuba tion period, in its high mortality, extremely cellular ascitic fluid and occasional solid tumors For its transmission the ascitic fluid must contain at least ten times as many cells as ordinarily found in the hepatitis ascitic fluid

Best Will you tell us a little about Ehrlich's tumor? It is a new one to me

Minck It was a new one to me until I began searching around to find out what this disease might resemble It is being studied rather extensively at the Sloan Kettering Institute by Dr Sugiura who gave us the strain that we have worked with It was first described as a mouse carcinoma by Ehrlich and has been found to be readily transmissible in mice In 1932, Lowenthal and Jahn obtained a special variant of this carcinoma which usually produces after intraperitoneal injection no solid tumor but rather huge ascites of a milky or bloody character This ascitic tumor is being used quite extensively in studying the possible chemotherapy of neoplasms

The agent that is the agent of this hepatitis we are describing is thought to be a virus but when compared with the known virus diseases natural to mice it seems to be identical with none Pneu monia virus of mice (PVM) is excluded by the viscerotropism of the agent and the absence of hemagglutination the psittacosis

group by the absence of inclusion bodies, avirulence to embryonated eggs, and the absence of specific C F antibodies in hyperimmune rabbit or convalescent mouse sera The hepatitis described by Olitsky and Casals (21) and others is excluded by the absence of inclusion bodies, evidences of clinical disease, and the possibility of transmission

Our disease differs from the hepatitis of mice recently described by Gledhill and Andrewes (22) in several respects Unlike our agent their virus is reported to infect only suckling mice, or weaning mice of one strain (BSVS of Webster), it has a short incubation period and is highly lethal for suckling mice The lesions produced by the two agents though somewhat alike microscopically, are grossly dissimilar

Some strains of lymphocytic choriomeningitis virus may produce a disease similar to that which we have described (23) However, our agent has not produced meningitis when inoculated intracerebrally into LCM susceptible mice, nor detectable or transmissible disease in embryonated eggs or guinea pigs Mice convalescent from hepatitis or acutely infected with hepatitis were equally as susceptible to LCM infection as controls Moreover, our agent was not neutralized by hyperimmune anti LCM guinea pig serum, and hepatitis mouse spleen antigen did not fix complement with this serum It is noteworthy, however, that Dr Wallace Rowe has partially neutralized LCM intraperitoneal infection of mice with a convalescent serum pool from our mice, and this serum fixed complement with LCM guinea pig spleen antigen

Preliminary experiments with several chemotherapeutic agents are summarized in Table III It will be seen that streptomycin, terramycin, chloramphenicol, and sulfadiazine, in the doses indicated, for the entire experimental period, that is, beginning at the time the mice were inoculated intraperitoneally and continuing until the time they were sacrificed, did not seem to reduce the incidence of hepatitis or of ascites, as compared with the controls However, penicillin given intraperitoneally or orally seemed to inhibit the disease completely

These experiments I call preliminary, because the number of mice involved were obviously not sufficient to be too emphatic about the results

Madden Is this prophylactic or treatment?

Mirick This is prophylactic The penicillin was given the same day that they were inoculated, and the mice were sacrificed 28 days later They received penicillin throughout the entire period

TABLE III

Effects of Chemotherapy on Hepatitis in Mice

Drug	Daily Dose	Route	Time Days	No of Expts	Result Hep	Asc
Penicillin	100 u	I P	28	1	0/4*	0/4
Penicillin	350 u	P O	28	3	0/12	0/12
Streptomycin	500 u	S C	28	2	4/8	3/8
Terramycin	25 mg	P O	28	2	5/8	5/8
Sulfadiazine	10 mg	P O	28	1	4/5	3/5
Controls	0	0	28	3	10/11	5/11

* Numerator number of mice showing lesion Denominator number of mice inoculated

It has been tried in treatment and seems to have no effect at all that is, when started the third week after inoculation

Stokes Have you tried penicillin prophylaxis in humans?

Mirick I have not, Dr Stokes, because, as you know, I don't see very many people that I know are in the incubation period of hepatitis, but I think that is one thing that we ought to consider as a thing to do

Stokes Yes

Best I wonder why Dr Stokes asked that question Is this virus supposed to be the same?

Stokes As human?

Best Yes

Stokes I don t think Dr Mirick feels so I don t feel so from the studies in Philadelphia by the Drs Henle

Mirick I have been particularly interested in the apparent penicillin effect in relation to Gyorgy s (24) observations that antibiotics may suppress the development of dietary liver necrosis in rats Do you want to comment on that, Dr Gyorgy?

Gyorgy Penicillin will prevent it as well as terramycin and streptomycin

Watson Ordinary penicillin?

Gyorgy Ordinary penicillin by mouth procaine and dibenzyl penicillin

Watson I thought the dibenzyl penicillin was better

Gyorgy Slightly I don t think there is a great statistical difference

Best What do you think is the mechanism?

Gyorgy Well, I am prejudiced I still think it is the intestinal flora

Best How does that fit in?

Gyorgy I think maybe next year we shall talk about it

Hoffbauer Do you say prevent or delay the onset?

Gyorgy Well, you are right, we should say that the antibiotics have a delaying effect on experimental hepatic necrosis The delay may be often marked and may be synonymous with prevention

Mirick The effects of diet and cortisone on the mouse hepatitis have also been explored only in a preliminary fashion We have been unable to demonstrate any altered viral susceptibility in mice fed a diet containing 4 per cent or 10 per cent protein or a pyridoxine free diet Cortisone did not seem to enhance the infection

Best Why did you give cortisone to these animals?

Mirick To find out what it might do It might have made them better or worse If it made them better, it would make me believe that the animals themselves had some type of allergic reaction If it made them worse, that would fall in line with the effects of cortisone on other virus infection that have been studied

Our experiments with cortisone to date have been rather limited, and I can't say with certainty that it has no effect But, certainly, it did not block the disease once it had appeared That I can say Whether it makes them worse or not I am not prepared to say

Best You are really interested in the one thing you are studying, not just the effect of generalized hormones on the picture?

Mirick No I wanted to use it for the reason I stated to try to rule in or out allergic response and to try to enhance a virus infection if such were present

Best I was thinking you could take out the pancreas and probably enhance the infection couldn't you?

Mirick Perhaps so I have never thought about depancreatizing a mouse

Best Well you can give it alloxan To intensify an infection there is just as much reason for removing the pancreas as for giving cortisone At least, that is true for bacterial infection

Mirick Has that been studied in virus diseases?

Best I don't think it has ever been studied in virus diseases At least, I do not know that it has

Mirick You see cortisone has been rather extensively studied with viruses in a whole list of them that I went through and in each case it has been shown to enhance the disease

Best There is no doubt that the diabetic organism is terribly susceptible to infection i e bacterial infection

Mirick I think one cannot in any case reason from bacterial infections to viral infections They differ in so many respects

Best No except that you have a generalized thing such as the fact that any prolonged dietary deficiency makes animals more susceptible to almost anything

Mirick Except many viral infections

Best That is new to me

Mirick The exact opposite is the rule What I showed with chronic pyridoxine deficiency is the exception

Best That is very interesting

Mirick That was first pointed out by Dr Olitsky Guinea pigs that were sick or on deficient diets were less susceptible to infection by the agent of hoof and mouth disease than were normals That observation has been repeatedly confirmed with other viruses in other animals

Stokes Workers at The Children s Hospital of Philadelphia found a similarly increased resistance to poliomyelitis virus (Lansing type) in mice which had previously been made deficient in thiamine

Mirick The disease in the mice was not neutralized by homologous hyperimmune rabbit serum convalescent mouse serum anti LCM guinea pig serum human gamma globulin nor serums from patients convalescent from hepatitis Complement fixation studies with mouse ascitic fluid antigen have been positive in low titer with mouse convalescent serums and with certain serums obtained from patients at the peak of their hepatitis

Organ extracts and ascitic fluid infective for mice were inoculated orally and intramuscularly into 10 human volunteers No typical hepatitis with jaundice ensued All developed an illness compatible with serum sickness with some abnormal liver function tests They were observed and had weekly liver function studies for 65 days after inoculation Several continued to have a mild illness after 3 weeks Several developed slightly increased BSP retention and increased thymol turbidity values All developed positive cephalin flocculation tests and several positive thymol flocculation tests Several in addition showed a slightly increased excretion of urine urobilinogen They all developed heterophil agglutinins that is agglutinins to sheep cells rising from a titer of 1 60 before inoculation to a peak of 1 2560 at about the second week and maintaining a high titer during the period observed Three developed precipitins against mouse ascitic fluid These findings have been interpreted

as some evidence of liver damage but are probably attributable to foreign protein reaction or "serum sickness"

Sborov Were the heterophil antibodies absorbed on guinea pig kidney?

Mirick Yes, they were absorbed on guinea pig kidney

Kark Have these ten men been tested with Dr Stokes's skin test?

Mirick No, they have not We wanted to use the skin test, but unfortunately there was none of the antigen available

Stokes There is no reason why it could not be done later if adequate material is available

Hanger Where did you inject this material?

Mirick They received it in three sites each buttock and the deltoid in one arm

Hanger Over a period of time?

Mirick At one time

Gyorgy How much?

Mirick We gave quite a bit of material, because we wanted to have an all or none test They got a total of 10 ml of the material intramuscularly and 12 ml by mouth And the intramuscular material was half ascitic fluid and half a 10 per cent organ extract from the mice, which was bacteriologically sterile and was proven at the same time to be infective for mice

Hanger A very potent antigen, isn't it?

Mirick Yes, it seems to be

Gyorgy Do you have controls with normal mice tissue?

Mirick I do not I am not sure I know what normal mouse tissue is

Gyorgy That is true But at least not ascitic

Mirick No, sir, we have not done that

Popper Did you perform liver biopsies on the human volunteers?

Mirick We did not

Sborov Did anything happen after 60 days in any of these?

Mirick Nothing has happened We are now at about 86 days, and the last time I saw them was a week ago They were all feeling pretty good I did not do any tests on them then

Best When you say serum sickness do you include the possibility that there are unknown viruses in the serum, or do you mean some allergic reaction to the normal constituents of the serum?

Mirick What I mean is the latter I used the term not to imply any type of infection but an allergic reaction to foreign protein

Best Could there be some unknown viruses in this?

Mirick I presume there could be but we certainly failed to demonstrate that there are viruses which will give human beings typical, or even atypical, hepatitis of any severity

Watson Did you not feel that you had some evidence of liver functional derangement?

Mirick Well, I think that is true

Watson I thought so It looked that way to me

Mirick Several of the tests definitely became abnormal and then returned to normal That is particularly true of the cephalin flocculation, of the thymol turbidity, and of bromsulfalein excretion

Gyorgy Who knows what liver function tests should be used in serum sickness?

Mirick I don't believe anyone knows I looked for the answer in the literature but found nothing published on that subject I have asked a lot of my friends who are clinicians, but none of them knows the answer The statement is made that the liver becomes enlarged and tender in a small percentage of cases, 3 to 4 per cent of cases of serum sickness

Popper There is a difference between infectious mononucleosis and serum disease in the absorption of the heterophil antibodies If I understand you right, in your case the antibodies are of the Forssman type absorbed by guinea pig kidney, as in serum disease, in contrast to infectious mononucleosis

Mirick That is right It is not like infectious mononucleosis

Best So serum sickness means sickness from any tissue extract

Gyorgy Any foreign tissue

Mirick It is called serum sickness because that is what causes most of the clinical difficulty, but I think the term could be used to apply not only to foreign proteins but perhaps to other substances which might combine with serum, don't you Dr Watson?

Watson I think so

Mirick Certainly, the allergic reactions one sees with sulfonamides

Best It started as antigen and then serum and now it is carried on to any extract of any tissue

Mirick Or I think even anything that may hook up with protein Sulfonamide reactions are of the same type

Gyorgy Penicillin, too, Dr Watson?

Watson That's right It produces a disease which is often indistinguishable from serum sickness, and it is probably related to the binding of the protein

Best We are getting ourselves into difficulty in calling the effects of penicillin, serum sickness when that term refers back to horse serum

Gyorgy We may call it serum sickness because penicillin is tied up with serum proteins and becomes antigenic as any foreign protein

Mirick Certainly, in this case it is really not serum sickness It is ascitic fluid sickness

Best I think that clarification is needed When a set of symptoms from penicillin or mouse ascitic fluid are the same and not distinguishable from those of horse serum and all are called serum sickness, it is time to make a change

Hanger They are really called foreign protein reactions Serum sickness is one of the common forms of the phenomenon

Knisely They certainly should be separated

Mirick None of these patients developed the clinical picture typical of serum sickness due to horse serum In that reaction, the most common symptoms are rather brisk fever, general glandular enlargement, hives, angioneurotic edema, and arthralgia These symptoms usually appear within the first two weeks, usually around the tenth day, and very rarely do they appear after the third week None of these patients had appreciable fever during the first two weeks Moreover, none of them developed hives I mentioned pruritus, but not a single one of them had any hives None of them had angioneurotic edema The gastrointestinal symptoms were much more prominent than one ordinarily finds in serum sickness They were typical only in that they did have general glandular enlargement and some arthralgia

When I explain this by saying it is probably a type of foreign protein reaction I am merely trying to explain our findings in other terms than the result of an infection, but the disease was not the typical serum sickness described in man

Shorr May I ask a question, Dr Mirick? I gather that much of the discussion is related to the relevance of these findings to human hepatitis I wonder whether it is to be expected that a virus would under these situations, remain unmodified in passage, or whether you might not anticipate that some degree of modification would occur Under the latter circumstance your experiments would be valid with respect to diagnostic studies if not to their extension to include the passage of the virus back to the human

Mirick One would expect the virus to become modified and attenuated Dr Stokes can probably add some facts on that Had

I not expected that, I never would have dared to use the volunteers I would not have dared to inject ten men with the original liver we started with from a patient who died Dr Stokes, do you want to comment on the disease in man following embryo passage of your viruses?

Best Or would you rather keep that for your opening discussion?

Stokes All right, I will defer that until later

Popper I should like to ask about the significance of the heterophilic antibody agglutination, obviously the most characteristic finding in your patients To my knowledge, it does not occur in human viral hepatitis, at least not with any frequency (25) Some of our cases have been thoroughly studied by Dr I Davidsohn and he did not find positive reactions Would you tell us in what human disease, besides serum sickness or other foreign protein reactions, agglutination of sheep erythrocytes by heterophilic antibodies is found?

Mirick Infectious mononucleosis (but in that disease it is absorbed differently) and in some cases of leukemia I don't think of others that characteristically develop heterophil antibodies

Watson I don't think of any at the moment It certainly is not usually positive in infectious hepatitis

Neefe It is in a percentage of cases, I think At a center in Germany a whole group was screened a couple of years ago, and as I recall, some 15 or 18 per cent did have a positive heterophil but never over 1 to 56 (26)

Popper Were the antibodies absorbable by guinea pig kidney?

Neefe Yes

Popper Then it is not the same as in infectious mononucleosis

Neefe It is not the same as the antibody of mononucleosis

Mirick I think there is no doubt that these patients that I have shown develop antibodies against mouse protein All of that hetero phil antibody can also be absorbed with mouse tissue But the question in my mind is whether there is anything more than that Even though they do develop an immunity to mouse tissue or a reaction to it, may there be something else in addition?

Smetana Did you think these patients developed hepatitis or something comparable to hepatitis?

Mirick I think that five of them developed some mild form of hepatitis Probably if we knew the answer, patients with serum sickness develop hepatitis too But I don't think I would go so far as to say that it indicates that we have infectious hepatitis of any type although that is still a question

Smetana That is what I wanted to ask you whether the liver injury is automatically identical with hepatitis, particularly of the infectious type

Best You mean that horse serum gives an inflammation of the liver

Mirick I think it does, yes I can't substantiate that, but certainly the reaction to foreign protein has been shown to produce histologically detectable changes in many tissues

Best I think P P Laidlaw* did that first with pure protein and he found very definite histologic changes in anaphylaxis

Mirick This brings us back to the question of allergic symptoms as seen in the infectious hepatitis of man, described earlier What are the men allergic too, if those symptoms are to be explained on the basis of allergy? Two subsequent attempts to isolate the agent from the original human liver were successful, but recently, control series of mice inoculated with normal human serum have also developed the disease This suggests that the strain of mice employed may carry the agent

We sent the agent to a number of our friends in other laboratories several months ago I haven't their results at hand, but so far, without exception, they have been able to produce the type of disease described with the material sent them In some laboratories, they repeated what looked like isolation of the disease, by our method, from infective sera and not from normal sera In other laboratories, however they have found that any serial passage of mouse organs in mice results in the infection I think what it adds up to is that mice probably carry this agent though why it has not been previously noticed or described, I can't imagine

Another point that I should mention is that a few mice have spontaneously developed this infection that have not been inoculated but have merely been kept in the cages or the same room as mice that we have inoculated In other words, though our experimental efforts to transmit the disease by some other method than injection have failed, it seems evident that it may be transmitted in some way from mouse to mouse Our isolation has been far from perfect We have had the same people taking care of all the animals, and it is conceivable that the positive controls that we have found may merely indicate an inadvertent transmission of the agent to the control series

Watson Dr Mirick in that connection has this been described in any laboratory that has not had the material from you?

* Personal communication

Mirick Not to my knowledge, Dr Watson

Best You would think that mice would come down spontaneously with this thing, wouldn't you?

Mirick One would think so

Watson Would it be worth while to send a questionnaire to laboratories in which large numbers of mice are used to see if they have observed this from time to time? For example, John Bittner has had an enormous mouse colony for many years He could tell us right away whether he has seen this type of thing develop spontaneously

Mirick Some strains of lymphocytic choriomeningitis virus can produce, as I tried to indicate, ascites and hepatomegaly, a picture very similar to the one we have described, and I think unless someone were actually trying to rule out infection with our agent, he would assume that it was due to lymphocytic choriomeningitis In other words, I don't know what I would specifically learn from the questionnaire if I got positive answers

Watson I see

Mirick [illegible] e
available [illegible]
There is n[illegible]
ever, the disease described resembles hepatitis in man in some respects, and it may be a useful laboratory tool in studying the human infection

Whether or not the agent we have described is related to the cause of hepatitis in man, the experimental approach which assumes some second factor to influence the host's susceptibility to hepatitis warrants further testing The reasons why some human beings seem to be more susceptible than others to infection by the naturally or artificially transmitted disease are still unsettled It seems likely that the presence or absence of acquired immunity from previous overt or subclinical infection will not explain all of the facts

Best I shall ask Dr Stokes to open the discussion

Stokes I think perhaps I could best discuss the outbreak that Dr Mirick described by presenting one slide (Table IV), from which I can draw some conclusions and make some basic points that have to do with questions raised

Table IV presents a rough outline of an instance of epidemic or endemic hepatitis that we studied with Dr Capps and Dr Bennett in Chicago We had been studying it really from a distance, from Philadelphia Then Dr Capps' group in Chicago joined us in the study about the middle of 1949

TABLE IV

Endemic Hepatitis — Chicago Orphanage

1946	1948 Nov 7	1949 Sept 1	1951 Jan 1
139 N 44 J 32%		20 N 9(6 + 3)** 45%	
36 N 1 J* 3%	→	35 N 1 J† 3%	

* Given gamma globulin 2 days before onset
† Not exposed to infective children until fall 1949
** 6 jaundice 3 without jaundice

The orphanage in question had suffered from the endemic disease since 1942 and they were having difficulty in getting both interns and new nurses in 1948 When Dr Sauer, who was in charge of the orphanage, requested aid, I suggested that he use gamma globulin because there obviously was an endemic focus of infectious hepatitis The new student nurses had been coming down with hepatitis with jaundice Later we found it was unquestionably epidemic hepatitis

Only one of the children in this orphanage had had actual jaundice during the same period Between 1946 and 1948, out of 139 nurses, 44 had jaundice, or 32 per cent

Gamma globulin was started on November 7, 1948, and of the 36 new nurses who were injected 0 06 ml per pound body weight, within about one month after admission to training, one developed jaundice, indicating that the virus was still endemic in the institution She had been given gamma globulin only 2 days before the onset of the jaundice In other words the incidence was 3 per cent, so that the gamma globulin in that amount was highly protective

In order to study the situation further, because we had to determine where the focus lay on September 1, 1949, the injections of gamma globulin were stopped, following which 20 new student nurses were admitted In the next 3 months 9 developed hepatitis, or 45 per cent whereas of the 35 nurses who remained from the

original group injected with gamma globulin only one developed hepatitis or 3 per cent

Both groups of nurses (injected and noninjected) were in contact with each other and in contact with the same children except the one injected nurse who had developed jaundice It so happened that she had not been exposed to infective children in the fall of 1949 In other words after she had had gamma globulin in all probability she had not been intimately exposed to the virus of epidemic hepatitis

It was found that the association of the nurses with the children particularly on the fourth and fifth floors of the orphanage was apparently responsible for the disease Dr Capps Dr Bennett and their technical group at St Lukes Hospital in Chicago carried out extensive studies on the children finding positive liver function tests in about 40 to 45 of them We obtained stools from two children that had had symptoms for 5 and 15 months respectively with evidence of liver dysfunction Preparations from these stools were ingested by volunteers in the Philadelphia area who developed hepatitis with jaundice In other words we demonstrated that the children with chronic symptoms had the virus in their stools

It then became important to determine how the nurses could be protected It was found that they did not use adequate techniques in handling the children and as a result of simple changes in the techniques there has been no case of jaundice in this institution from January 1 1950

The reason I reviewed this situation in some detail is that it brings up important points in relation to the whole field of epidemic disease This endemic disease had an incubation period of 30 days as most epidemics that we have studied have had During this last year with the help of Dr Alexander Langmuir we have been notified of many epidemics throughout the United States and Dr Drake in particular has studied several of them in Missouri in Georgia and elsewhere in the United States In all of these outbreaks the incubation periods have been approximately 30 days

In addition to the relatively constant length of the incubation period I want to emphasize several important points The first is that the gamma globulin is highly protective in epidemic hepatitis and we now have reduced the dosage to 01 ml per pound body weight which means that in a 200 pound man for instance only 2 ml would be protective This is lower than the amount that is protective against measles for instance Experiments conducted at the level of 0 005 ml per pound body weight also indicate protec

tion at that level apparently, although more extensive studies are required In other words, we feel that the lower level may be around 0 005 to 0 01 ml per pound body weight

Best This is gamma globulin from unselected blood donors?

Stokes Yes, from the Red Cross pools in the United States

The second thing I want to point out is that the nurses who had been injected with gamma globulin during the summertime may have obtained a passive-active immunization They were passively protected first by the globulin Then, apparently, the nurses were being exposed to the virus in this institution continuously Although they were exposed for about three times as long as the new nurses who entered on September 1, still they did not develop the disease This strongly suggests that they had developed a passive active immunization, that is, the antibody first, the antigen second and we feel this is the most likely reason for their protection

In an institution outside of Baltimore, for retarded children and in one outside of Philadelphia, New Lisbon, for retarded males we have had similar exposures to antigen following the injection of antibody, which also strongly suggests a similar ability to develop a passive active immunization There are other possible explanations, one being that the gamma globulin itself might produce active immunization which we do not believe is probable, since a neutralized mixture containing virus and a large excess of antibodies is a very poor immunizing agent

Best How long does the passive immunity persist?

Stokes In these institutions, it persisted for 7, 8, and 9 months respectively In measles, we know that if a child is not exposed continuously during his passive immunity he becomes susceptible again in about 5 to 6 weeks after the injection of gamma globulin Since the half life of homologous antibody in man is only about 20 days it would be difficult to believe that the passive immunity alone could last over 7, 8 or 9 months

Best Is it possible to superimpose an active immunity to measles on the passive?

Stokes Certain work by Hermann in infants strongly suggests that There is also evidence for the presence of such a mechanism in certain other viral diseases Work in animals, particularly, is in full agreement with these findings

The carrier state, or a chronic active hepatitis in these children, is a very important third point Of course, susceptibles were being introduced into this institution continuously, so that is the way we feel the disease remained endemic The children whose stools we

tested had symptoms for 5 and 15 months respectively In these children evidence of chronic active disease was suggested by the presence of positive liver function tests

After epidemic hepatitis chronic grumblers not infrequently occur in individuals with a chronic active hepatitis These cases may develop fibrosis of the liver sometimes a mild fibrosis but at times an extensive fibrosis followed by ascites This has also occurred in serum hepatitis as exemplified in a child who developed the disease apparently as a result of the SH virus being transmitted from the mother through the placenta In this case we eliminated the possibility of the infant having the disease by transmission through the birth canal or through the mother s milk because the baby was delivered by Cesarean section and was separated from the mother until about the time that active hepatitis developed The baby died at 18 months with chronic fibrosis of the liver At 9 months we obtained blood both from the baby and from the mother and found evidence of hepatitis in 2 groups of volunteers one group being injected with the infant s blood and the other group with the mother s blood Two years and eleven months later we again tested the mother s blood in volunteers and in 2 out of 5 volunteers hepatitis with jaundice developed in approximately 75 days We thus showed that she was a carrier without a history of jaundice and without positive liver function tests in other words what might be called a silent carrier

We have been studying what apparently are two additional carriers of the serum hepatitis virus one in England (from whom Drs Maycock Zeitlin and MacCallum have given us blood) who had transmitted the disease to recipients of his blood over a period of about 15 months while the other carrier in Philadelphia has transmitted serum hepatitis over a period of 5½ years to recipients of his blood The latter is a chronic alcoholic and has positive liver function tests and evidence on biopsy of a chronic active hepatitis He was first described by Drs Berks and Malamut Neither of these carriers has had a history of jaundice

Dr Neefe can speak of others that he has been studying with Drs Norris Oliphant and Murray

It is important to distinguish between chronic epidemic hepatitis (IH virus) as seen in the Chicago orphanage and the chronic active serum hepatitis (SH virus) as seen in the adult carriers just mentioned We know of only two individuals the two children mentioned who may be considered as chronic carriers of the virus of epidemic hepatitis the agent being excreted in the stool There have

now been demonstrated about 7 cases of chronic carriers of the serum hepatitis virus, but the virus has not been found in the stools of these individuals We have even taken filtrates from the stools of cases with serum hepatitis and, after filtering, injected them parenterally into volunteers without producing the disease, so that in the studies thus far, the virus apparently has been absent from the stools

This leads directly into a fourth point, namely, the question of fecal or oral spread of the disease The evidence of the presence of virus in the stools of the two children that I mentioned, the evidence that the disease stopped abruptly when the nurses' techniques were altered, and the evidence that a large group of pregnant women who were delivered in the orphanage and exposed to the same personnel as the student nurses were, with no cases of hepatitis with jaundice among them over the period from 1942 up to the present time (except one mother who developed it about a week after she came into the orphanage), all indicate that there was probably no respiratory spread of the disease It would be hard to believe that in such an institution, if the disease were spread by the respiratory route, that over a period of 8 years a few of these mothers would not have had the disease

Dr Neefe and I some years ago in a large outbreak in a summer camp, obtained secretions previous to, during and after the active disease With Dr Drake, we have repeated this study recently because we have continuously been impressed with what Dr Mirick mentioned, namely, that a fair percentage of individuals during the incubation period develop respiratory symptoms We have taken secretions from these individuals and have attempted to produce the disease Both studies in volunteers were unsuccessful in demonstrating the presence of virus

One must consider the possibility that the presence of the hepatitis virus in the blood and elsewhere during the incubation period may lower the resistance of the infected individual so that other viruses to which they are exposed may be able during that period, to cause mild respiratory diseases Also, the possibility of an allergic response must be considered since there may be such responses during the early phases of the disease In addition, some workers who are interested in viral diseases of the respiratory tract feel it may be possible that some of these viruses, like the herpes simplex virus in the skin, reside in the mucous membrane and that the lowering of resistance produces a sudden recurrence of active symptoms in much the same way that herpes simplex will occur

as a result of a digestive disturbance In other words there are several possible explanations for the respiratory symptoms and their presence would not necessarily indicate that the virus of hepatitis itself is causing the actual symptoms

In the Chicago orphanage at least the fecal oral route seems to be a clear cut route of transmission as it has been in other situa tions In the families we have studied in connection with many of these epidemics it seems to have been spread in the same way the fecal oral route And in food milk and water quite frequently the virus is present In other words the virus appears to be trans mitted in much the same way that typhoid fever is transmitted in water food and milk and by direct contact

A fifth point is also of great interest in this epidemic namely that the jaundice was present in adults and not in the children under 3 years of age In this and in other epidemics we have noted a direct relationship between the length of jaundice and the age Generally speaking the older the individual the longer the jaundice while the younger the individual the shorter the jaundice And when one studies the incidence of jaundice in children under 3 years a very large percentage of the cases we believe occur as hepatitis without jaundice They frequently have ordinary gastrointestinal symptoms and particularly diarrhea

As a sixth point Dr Drake and I determined jointly with Dr Capps and Dr Bennett the presence of positive skin tests in those nurses that had had jaundice as well as in the majority of children on the fourth and fifth floors which represented the endemic focus In the newly admitted children s receiving ward only approximately 10 per cent of the children had positive skin tests whereas about 90 per cent of the nurses who had had jaundice had positive skin tests with the material used (The skin test material was virus from an epidemic in a Pennsylvania children s camp grown in the amni otic sac of the chick embryo following repeated passages by the Drs Henle at the Children s Hospital of Philadelphia The infected amniotic fluid is irradiated with ultraviolet light)

When Dr Drake and I carried out the skin tests Dr Bennett and Dr Capps assured a random sampling by changing the locations of the patients in the orphanage including those in the receiving ward so we would not know which children had had the disease and which had been on the fourth and fifth floors The correlation between those that had had the disease including the nurses and the positive skin tests appeared to be very good We have found similar correlations in other epidemics We have done many thou

sands of skin tests, and there has been a good correlation between a history of epidemic jaundice and positive tests However, this is a difficult material to preserve even under lyophilized conditions, and different batches differ considerably in their activity These are the reasons it has not been more generally available A correlation has not been demonstrated between positive tests and serum hepatitis with jaundice

A final point is worth mentioning The protection obtained by means of gamma globulin in infectious hepatitis also helps to differentiate between the serum hepatitis virus and the epidemic hepatitis virus We have continued to experience complete success in affording protection against epidemic hepatitis with gamma globulin, as have many others during the last year On the other hand with convalescent gamma globulin from serum hepatitis cases we have been unable, either through neutralization tests or through more than one or two injections, to protect the volunteers against the serum hepatitis virus of the strains we have used When one combines these findings with those relating to the lack of cross immunity between the strains of IH virus and SH virus studied, it seems clear that these viruses have marked antigenic differences

It may be worth while to mention two points One of these, shown in Table V, relates to the effect of gamma globulin on serum hepatitis virus This is an old piece of work, but it illustrates what we have found to be true since that time Two milliliters of infective plasma were injected into 4 volunteers, 2 of whom developed hepatitis and jaundice with long incubation periods as shown

The next experiment was a mixture of 2 ml of the infective plasma with 1 ml of gamma globulin, incubated at 37° for 1 to 2 hours, sometimes 1 and sometimes 2, and kept in the icebox over night There again 3 out of 5 had hepatitis with jaundice with long incubation periods

In the third experiment, 2 ml of infective plasma and 10 ml of gamma globulin were given simultaneously at separate sites

We have not only repeated this but have increased the gamma globulin to 20 ml this together with 1 ml of infective plasma in a neutralized mixture has shown no neutralization

Recently, as I have mentioned we have obtained plasma from individuals convalescing from 3 months to 3 years from the Ft Bragg strain of serum hepatitis virus Sharpe & Dohme has fractionated it for us by the ethanol fractionation method of Cohn and Oncley (27) We have not been able to show any neutralization

TABLE V

Effect of Gamma Globulin on Hepatitis Virus SH
Experiment I

Injected into Volunteers	No of Volunteers	Hepatitis	Injection to Onset Interval (Days)
2 ml infective plasma	4	2	101, 112
Mixture of 2 ml infective plasma and 1 ml gamma globulin	5	3	105 117, 136
2 ml infective plasma and 10 ml gamma globulin simultaneously at separate sites	5	2	108 112

Reprinted by permission from Blanchard M C *et al* Methods of protection against homologous serum hepatitis the inactivation of hepatitis virus SH with ultraviolet rays *J A M A* 138, 341 (1948)

with this material in volunteers whether given at the time the infective plasma was injected or 30 days later

In relation to the antigenic differences and similarities, there has been evidence of a relationship between a positive skin test and experience with the epidemic virus in all epidemics thus far studied in this country Because these relationships have included epidemics widely separated in time and space, we believe that the evidence strongly suggests this virus is like the viruses of mumps and measles namely, of a single antigenic strain or type The evidence is not sufficient to suggest such an antigenic unity with respect to the serum hepatitis virus or viruses

Dr Drake has obtained strongly suggestive evidence that the *skin test* antigen of the epidemic virus that I have mentioned has produced at times an active immunity There have been three outbreaks in different sections of this country where the skin test [illegible]

ties as a skin test disappear whereas with the mumps antigen, if one dilutes it even up to 1 to 100, it continues to be active as a test

But this also suggests that the method used to *irradiate the IH* virus in amniotic fluid is not entirely successful in inactivating the

virus It might be diminished in amount or it might be considered as attenuated, and still produce an immunity in the individuals injected

The Akiba strain of virus, from which this skin test material was obtained, infects quite readily when ingested but infects with some difficulty when injected parenterally This may account, to some extent, for the fact that when it is used as a skin test, it does not readily produce disease, even though it may still be active

In connection with Dr Mirick's discussion when the infective yellow fever vaccine was given at the beginning of the war, despite the close exposure of a large number of individuals to those that had hepatitis with jaundice, there were only four instances that could be found in which men who had developed the disease at home had transmitted the disease to members of their families I think the four wives who were infected appeared to have contracted the disease from their husbands I believe they had long incubation periods This has made me wonder about the possibility of venereal transmission, as well as, perhaps, transmission through the placenta Also the serum hepatitis virus may well be transmitted by biting insects I don't believe that the experience with the yellow fever vaccine would suggest that the SH virus was transmitted through the respiratory route

The Drs Henle have conducted studies with Dr Mirick's virus and have attempted to neutralize this virus with three specimens of gamma globulin which were proven to be protective in epidemics of infectious hepatitis Neutralization did not occur with any one of these three specimens of gamma globulin This agrees with Dr Mirick's suggestion, namely that this is a mouse virus and not a virus actually from the case of Bauer, the pathologist who died

Hanger Dr Stokes brought up something that is clinically of very serious moment We are having more and more posttransfusion jaundice in hospitals and coincidentally, our sources of blood are coming more and more from commercial blood banks which are located near the slums of the cities, particularly here in New York Many of these professional donors I imagine spend their leisure in the grog shops I am wondering whether in other cities also it has been noted that as the donor becomes lower in the economic scale he is more apt to be a chronic carrier of the virus

Best Are you suggesting that the alcohol damages the liver?

Hanger Yes alcohol and the poor hygiene of many of these fellows who get some of their livelihood from selling blood

Best I was interested in that same point because Dr Stokes said his irradiation technique did not destroy this virus

Stokes I think obviously whole blood cannot be irradiated

Best No but the plasma

Stokes The success with plasma I think depends upon several factors number one the titer of virus that is present in the plasma Of course another is the amount of irradiation that is used Those different factors have not been evaluated as yet Some plasma is known to be infective For instance experiments by Oliphant originally and by our group indicated that certain types of irradiation could inactivate the virus Recent studies which I am sure Dr Murray would be willing for me to mention indicate that two of the methods have not been successful with a highly infective pool that they have been studying For instance the Dill apparatus has not been successful also a single passage through the Habel Sockrider apparatus has not been successful When it was repeated several times the virus apparently was inactivated

Best Could you safely irradiate cells if they were protected in glycerine?

Stokes I dont think anyone has determined that at least not to my knowledge

Tullis How about the contamination of the transfusion equipment as a source of future inoculation? For instance it used to be a custom in the services where there were long lines of people to be immunized at a time to use one syringe and multiple needles for a series of inoculations In December 1948 the Bureau of Medicine and Surgery recommended that no equipment used to puncture the skin for any purpose be re used without proper heat sterilization Such precautions have become standard practice in naval hospitals Presumably it is the material in the syringe that carries the virus It is the plasma or the substance in the syringe rather than the syringe itself

Hanger These are transfusions I dont believe that the modern blood bank is careless enough to permit contamination I think that more of our donors are carriers of the virus and it is just a question of whether their mode of living affects the carrier status

Stokes One of the important points is that none of the seven carriers of whom we are aware these include the ones Dr Neefe has been studying with Dr Murray and Dr Oliphant have had a history of actual jaundice There must be many hundreds or even thousands of such carriers

Best Do any of the viruses go through in Cohn fractionation?

Stokes The gamma globulin is apparently quite safe We tested that recently in Utah in a study of its value for protection against poliomyelitis

Watson The albumin is safe, too, isn t it?

Stokes All albumin that is used is heated in this manner and we have shown that is sufficient to inactivate the virus

Smetana Is it correct that if the plasma is kept at room temperature for six to nine hours the incidence of serum hepatitis is markedly reduced, in comparison to freezing or keeping the plasma in the icebox?

Stokes A study of that is being carried out rather extensively by Dr Murray at the present time in connection with the work that Dr Allen (28) of Chicago published Some of us, including, I believe, some members of the National Institutes of Health, are disturbed about the possibility that in small hospitals such serum or plasma, when held in this way, may be more readily contaminated The disease or deaths that may occur from contamination in small hospitals may be greater than any danger from hepatitis. The ordinary plasma that is not highly infective is helped by irradiation, but there should be enough irradiation to inactivate the virus in plasma of high titer

Smetana What is the rationale? Is it an enzymatic action?

Stokes The inactivation may well be similar to that of many viruses when held at room temperature Their inactivation is probably enzymatic in nature

Best It is worth while going on irradiating plasma, is it?

Stokes I think the ordinary plasma that is not highly infective is probably greatly helped by irradiation

Popper The question has come up as to the danger of transmitting homologous serum jaundice if the hematologic technician uses the same lancet without heat sterilization for several patients Should we use disposable needles for each count? If we were to go over to a more complicated method it would be a great expense for a large hospital like ours What is the risk in staying with the old method?

Stokes Well I think most of the clinical men here would agree that the disease is transmitted by using the same needle or instrument

Hanger It is not much of an expense to have a thousand or so needles which can be cleaned sterilized and used again and again

Popper Do you have them in your institution?

Hanger Absolutely

Stokes We use entirely different ones

Popper For each blood count?

Hanger For each blood count

Popper We have in our hospital a considerable incidence of homologous serum jaundice, possibly due to the fact that we have low income class donors and patients Would you, therefore, recommend that we should go over to an individual needle method?

Stokes That is my belief, and I think most clinical men agree with that

Mirick Dr Ratnoff and I tried to evaluate the importance of that factor by examining patients in retrospect We analyzed all the cases of hepatitis that had been admitted to The Johns Hopkins Hospital over a 12 year period We found that one out of every seven patients hospitalized with acute hepatitis had been transfused with homologous blood or a blood fraction five weeks to five months before the onset of the hepatitis Moreover, 20 per cent of 183 patients with hepatitis had had a parenteral test or injection of material other than blood or arsenic within five weeks to five months before the onset of their illness Only 8 per cent of a similar number of control patients without hepatitis had had a similar test or injection By control patients, I mean the next admission to the ward The probability that the difference noted could have occurred by chance is less than one in one hundred We concluded that this was definite evidence that the casual needle prick did account for a good number of cases of hepatitis We are using individually sterilized needles or knife blades exclusively at Johns Hopkins at the present time, even for doing blood counts

Popper Is this information published? It would enable us to discuss the matter better in view of the expense involved

Mirick We published that, yes It is in the *Bulletin of The Johns Hopkins Hospital* (29)

Watson There was another paper published recently with respect to the hazards of homologous serum jaundice for the medical technician

Popper Yes that was published in the *American Journal of Clinical Pathology* (30) I was thinking not only of the technicians but also of the patients, especially in a place where a large number of blood counts are made daily

Watson Yes but it is just as good evidence, at least

Kark Our allergists are resistant to the idea of changing their needles or using fresh needles We have had quite a time trying to persuade them to change over to fresh needles for every patient

Hanger Dentists also probably disseminate hepatitis We have had several patients with the disease who have had no known exposures except visits to dentists a month or more previously

Best I remember a survey that our bacteriologist made in Toronto of ophthalmological instruments and those used by the nose and throat people The protest was that if sterilized properly, the instruments would fall apart so therefore it couldn t be done

Hoffbauer Perhaps that is the reason that the dentist doesn t heat sterilize his instruments

Hanger A dentist cannot work in gloves and it is extremely difficult to wash his hands and clean certain instruments adequately between patient examinations

Rappaport I have seen a practical solution for hospital ad ministrators Womens College Hospital in Toronto uses a simple piece of glass They break a piece of glass and have a very fine sharp point to puncture the skin It is excellent I had it used on myself and it doesn t hurt at all After use the glass is thrown away

Best I thought everybody used the glass Was this new to you when you came to Canada, Dr Rappaport?

Rappaport Yes

Best It is certainly old in Toronto

Watson I think most people don t use it

Best The little ends of broken slides are sharp We used them as students

Rappaport In Europe they use a special instrument the Frank Peter needle

Popper It is still used in Chicago

Hoffbauer The cost of a thousand needles is far less to the tax payer than the cost of hospitalizing one patient with serum hepa titis for four weeks or longer

Popper I recently had the privilege of seeing a few liver slides from what appeared to me an interesting study carried out in Dr Hangers laboratory Its nature was similar to what Dr Mirick has just reported May we ask Dr Hanger to tell us about it?

Hanger This is the work that Dr Melcher and Dr Regan are going to report in Atlantic City next Monday the same qualms arise which Dr Mirick had during his mouse studies Their work was done primarily on rats who had been treated about two weeks with cortisone and then were injected with the serum which you Dr Neefe had found contained an icterogenic agent These rats began developing focal necrosis in the liver about a week after

the administration of this serum Dr Smetana and Dr Popper have seen the sections and have noted several disturbing things about them One is that the lesions are not very cellular contrasted with those of human hepatitis But I think animals on cortisone might not develop an inflammatory reaction to the same degree that an untreated animal would The animals on cortisone alone have not developed lesions neither have those given the heated material The lesions are sterile Whether the agent is a human or animal virus is yet to be determined Four consecutive passages of this infection have been obtained

Smetana I have seen these slides and as Dr Hanger says there is a lesion but it is not comparable to human hepatitis The other disturbing factor is that similar lesions are seen in other organs particularly the spleen

Mirick Of course I dont think we should expect necessarily when someone succeeds in adapting this virus to an animal to find exactly the same pathologic picture that one does in man

Popper That is true

Mirick The simple analogy is experimental influenza which produces a fatal pneumonia in a mouse and as far as I know rarely if ever does in human beings

Smetana But of course to make the cycle complete you want to have Kochs postulate fulfilled That has not been done as yet

Best Which postulate?

Smetana Kochs postulate to produce it again in human beings

Kark In how many diseases has that been done? We accept specific organisms as etiologic agents in many diseases without fulfilling Kochs postulate For example has it been done for typhoid fever?

Popper No it has not To my knowledge no animal with the exception of the chimpanzee can be infected with typhoid fever There is a hereditary immunity

I want to add to the remarks of Dr Smetana that the lesions in these cortisone-treated rats look like the result of a vascular occlusion They look like local anemic infarcts in the rats of Dr Hangers group Also in one of the pictures of Dr Miricks mice the circumscribed necrosis possibly may be the result of a vascular occlusion As it has been rightly said an adapted virus may produce a different picture and attack different structures

Smetana It is again in my opinion a question of diagnosis If you are satisfied clinically that it is hepatitis in mice then it is all right

Mirick I am satisfied, if you are speaking of our infection, that it is hepatitis in mice, but that isn't saying very much That doesn't mean that it is the hepatitis of man in mice

Smetana I mean comparable to infectious hepatitis in man

Mirick I wouldn't go that far

Popper Would, in Dr Mirick's cases, liver biopsy on the volunteers not represent a crucial experiment? We all feel that the chemical findings are not characteristic for viral hepatitis but that the histologic picture of the liver, though not definitely diagnostic, can be strongly suggestive for viral hepatitis

Mirick There are a number of things that might be done further with the volunteers, namely, to challenge them with known hepatitis virus and see whether they are immune to pass their acute phase serum to other volunteers and see if the virus would become readapted to the one producing the typical disease in man, and to try to neutralize the disease in mice with the convalescent sera from the volunteers I prefer the latter test since it does not involve further work with the volunteers Whether the others are worth doing or not, I have not decided

Stokes We have always had to avoid liver biopsy, because one loses volunteers immediately if one starts liver biopsies

Mirick I have avoided it, I think we all do, because of the conditions under which it would have to be done, for one thing A prison is not a hospital That is at least true for the type of volunteers we have nowadays

Best Any other discussion?

Tullis I would like to ask a question on immunity, a different subject You mentioned Dr Stokes that the gamma globulin seems to produce a passive active type of immunity in these patients and also you mentioned that children under three years do not get the disease [illegible] endemic I was wondering whether any childre[illegible] say, at the age of two years [illegible] of time, and developed hepatitis after the three year period, say at their fifth year, or did they, too, get passive active immunity?

Stokes We believe that most of the children did develop the disease under three years while they were there

One of the interesting points about the skin test in the orphanage was that there was an increasing percentage of positivity as related to the length of time that the child was in the institution That they did not have jaundice was our point In other words, Dr Katz and Dr Bennett in their liver function tests, indicated that about

40 to 45 of the children had positive liver function tests among those that were chronic "grumblers as one might call them For instance in the children whose stools we tested five and fifteen months after the onset of disease positive skin tests developed after a long period of the grumbling disease You misunderstood me that I said they did not have hepatitis We believe that practically all of them finally contracted hepatitis

Kark I want to go back to the very beginning of the discussion You were implying Dr Mirick that there were precipitating factors in the development of infectious hepatitis I wonder what other people think about it Nobody has discussed that at all I am thinking of much the same things as occur in polio the suggestion that operations on the ear nose and throat precipitate polio that fatigue or overexertion precipitates polio What about infectious hepatitis?

Mirick I should like to hear comments on this point The only fact I actually brought forth was the apparent influence of respiratory infection during the early part of the incubation period in producing a higher incidence of hepatitis in people who had been inoculated with the virus

I have no other facts to contribute but I should like to hear what others think about environmental factors or concurrent infection in increasing susceptibility or likelihood of developing the disease

Watson I have not had any reason in my own experience to believe that there was any precipitating factor As I think back over cases I have seen I have not been impressed by any greater incidence of respiratory infections or of anything in the immediate background prior to the development of the disease If there is such evidence I have missed it I have not been aware of it

Hartroft Do you include cases of alcoholism in this series?

Watson Yes I do

Davidson I suppose that with every chronic alcoholic who comes into Boston City Hospital with jaundice the intern makes the query is this infectious hepatitis superimposed upon a so called alcoholic liver? I had always thought there was no reason to believe that it was until recently The more biopsies we have done the more we have been impressed that there is a group of patients who may not have the classic picture of a fatty liver or a fibrosis or what have you who are chronic alcoholics badly nourished come to the hospital deeply jaundiced and have a different pathologic

picture in their liver from what we usually expect I don't know what it is

Smetana We have seen these lesions and we are very much interested in them It certainly is a distinct picture However, we have associated it with the alcoholic history rather than with hepatitis We have considered it the necrotic phase in the development of a portal cirrhosis of the liver

Popper We probably see a similar material at Cook County Hospital as at Boston City Hospital We have been very much interested in the patients with large fatty liver who die with severe jaundice from acute hepatic failure before cirrhosis has fully developed We have, of course, always looked for the cause of the hepatic failure I believe you are referring to the same condition

Davidson Not all of ours are stuffed with fat They may have a variable amount of fat Some of them look like omentum because they are so fatty, but others have just a moderate increase

Popper If the fatty metamorphosis is moderate, then it probably represents a Laennec's type of cirrhosis where the hepatic failure may originate from the cirrhosis

Davidson Well, no fibrosis I don't know what we mean by Laennec's cirrhosis

Popper I am referring to cases where the lobular pattern is replaced by nodule formation The cases we observed in this stage had a severe fatty liver, something that you described as looking like omentum without marked disturbance of the original lobular pattern We have now collected quite a large series of such cases and we do not know from what they died Their histories usually mention severe malnutrition or dysnutrition One of our last cases* was a young girl, a severe alcoholic, who worked in a candy factory and probably ate mainly carbohydrate and little protein Another case was a pseudohermaphrodite who lived alone, cooked his own meals and suffered from severe malnutrition

Histologically the picture is characteristic As Dr Smetana just mentioned the liver is riddled with small focal necroses, characterized by the presence of neutrophilic segmented leukocytes No histiocytes or round cells are found in contrast to viral hepatitis Moreover the necroses involve not one or two cells (as in viral hepatitis) but small groups of cells They are similar to what is usually described in acute forms of cirrhosis for instance by Volwiler Jones and Mallory (31) We therefore feel that morphologically there is no evidence for viral hepatitis in these livers except

* Observed by Dr F Steigmann and Dr W B Buckingham

would assume an adaptation of the histologic reaction due presence of fat That of course can be neither proved or ed Would you agree?

ana Yes

Experimentally, both in dogs and rats, there can be failure liver from fatty infiltration with no sign of fibrous tissue

ana And also in human beings

I mean in a deliberate experiment where there is just fatty ion If it is done in the right way, there can be complete of liver function before there is any fibrous tissue at all

bauer Is that associated with any histologic evidence of s?

No necrosis just fatty change

rlow The first case you described sounds just like what we West Indies call a sugar baby These seem to me to die just aving a fatty liver and nothing else if the liver contains han about 40 per cent of fat (on a fresh weight basis)

In the diabetic dog there can be a fatty infiltration no no necrosis and the insulin dosage goes down to zero and he animal dies He has no fibrosis and no necrosis just an im like liver

ier Are these dogs jaundiced?

Sometimes but it isn t a feature

ier We do not know what the cause of jaundice in these s Grossly the liver is green and very heavy weighing up pounds Would you agree with this?

ana Yes

ier They are amongst the heaviest livers we see Their is smooth Fibrosis is just beginning Pseudolobules and s have hardly formed Histologically severe cholestasis is

son Just one comment with respect to the cases Dr Davidson ned We have certainly seen a number of individuals about we wondered whether an infectious hepatitis had been added alcoholic fatty liver and in whom we were unable to be that that was the case But I think one should bear in mind hese alcoholics are subject to sanitary conditions that are ery bad and it would not be surprising if they were to pick virus of infectious hepatitis We cannot necessarily say that oholism per se is the precipitating factor In other words oholic under the conditions in which he is living may easily us hepatitis perhaps more easily than the normal individual

Popper In the fatal form of viral hepatitis, the portal lymph nodes are very much enlarged, as again Lucke and Mallory (15,16) have described These cases of fatty liver do not have enlarged portal lymph nodes

Kark Are you talking about people who die?

Davidson Yes

Stokes I think that there is not as much virus needed to kill such a person

Popper That may be

Best Is there any evidence that something aside from the virus, such as fatigue, increases the susceptibility?

Sborov Does malnutrition increase the susceptibility to virus disease or does it work in the opposite direction?

Best Dr Mirick told us that it worked in the opposite direction

Mirick I don't believe that there is any generalization that will hold I have looked, to some extent, into the nutrition work on viruses Of course, there are many viruses, many hosts, and many dietary factors to be considered In the majority of instances deficient animals have seemed to show increased resistance to viral infection but there are exceptions to that The most notable that I can think of is that thiamine deficiency seems to increase the susceptibility of pigeons to ornithosis and Dr Bodian showed that a strain of polio virus which ordinarily would not infect the monkey by oral inoculation was transmissible by that fashion in monkeys receiving desoxypyridoxine, or in pyridoxine-deficient monkeys But in most instances it is the other way around

I believe that in poliomyelitis – I should like Dr Stokes to comment on this – there are environmental factors which increase the severity of the disease

Stokes A deficiency of thiamine will increase the resistance of mice to the Lansing strain of poliomyelitis, also in encephalomyelitis of mice, resistance is increased by thiamine deficiency Severe strains of various sorts, such as cold muscular exercise, pregnancy, increase susceptibility to poliomyelitis So I don't think one can generalize about such factors

Best On the authority of an American who is accepted almost universally in Canada as a very great American, President Roosevelt muscular exercise and cold increase susceptibility to polio I was talking to him, the first time I ever met him, and he said Dr Best, I got polio by swimming in the cold waters of Herring Cove

I looked rather skeptical, and he said "You don't believe me?"

I said, "Well, I don't think it's quite like that"

He said, "I was swimming for a long time, and I got cold The muscular exercise and the cold lowered my resistance, and the virus, which was already present, took hold of me Isn't that a good theory?"

I said, "Yes sir, I think it is"

Hanger May I ask Dr Stokes whether, when he injects volunteers, he has any criteria as to which will be susceptible to infection and which will be refractory

Stokes No, I don't think we have any indication as to what makes a volunteer susceptible

Hanger Age, or habitus, or state of nutrition, or past alcoholic history?

Stokes Those that have a history of serum hepatitis are apparently more susceptible to the epidemic disease, and vice versa Age is a factor in that the older one is the more immune to the epidemic hepatitis virus That appears not to be true in serum hepatitis But as far as habit is concerned and other factors, I don't think we have, so far as I know, any indication of susceptibility in certain types of individuals

Hanger There is one other question I should like to ask you, dealing with reinfection Have you seen many patients who have gone into endemic hepatitis areas, have contracted the disease, become apparently cured, and then gone back to that area and become reinfected? I have seen a few where we presumed reinfection but we could not be sure

Stokes We have not had any experience with that type of situation to my knowledge

Neefe One does not often have the opportunity of studying persons who presumably have had two completely different attacks of jaundice (that is an apparently complete recovery from the first followed one or more years later by another attack) during the active stages of both attacks I have luckily had this opportunity on several occasions and in each instance different etiologic factors were involved in the two attacks We ran into this sequence several times in following up the summer camp epidemic group We knew these persons had had acute viral hepatitis (IH type) at the camp in 1944 followed by recovery On several occasions in the following years someone would call and say the youngster had jaundice all over again In one instance the boy clearly had mononucleosis as the explanation of the second attack of jaundice Another of the

boys had had a plasma transfusion and he had serum hepatitis (SH type of hepatitis)

Many times there are etiologically different processes with the exception of those who never recover completely from the first attack These persons if carefully studied would be found in many instances to have a continuous hepatic disturbance although perhaps at a subclinical level

Smetana This is something I wanted to ask whether reinfection can be clearly differentiated from recurrence

Neefe No I don t think it can be in the majority of cases Most persons once the original jaundice subsides have no subsequent follow up liver function studies Then sometime later an illness or recurrence of jaundice may appear In such persons one never knows whether they have actually had a complete recovery from the first attack or whether it was simply an apparent clinical re covery with subsidence of jaundice but persistence of subclinical liver disease I don t know of any way to prove this because most patients have not been carefully studied during the so called healthy interim Liver biopsy however may reveal changes of a chronic type with superimposed acute phenomena

Smetana Is it not possible that it remains inactive and can cause a recurrence if circumstances change?

Stokes That can be strongly suspected when epidemics of hepa titis are present One of the best studies of that sort in the field was by Gauld (32) in the Mediterranean theatre where he studied those men that had had serum hepatitis with jaundice as a result of the yellow fever vaccine In those individuals who had vaccine jaundice there was a higher incidence of the epidemic disease than in those who had not had the vaccine jaundice They were in the same division and the men were all associating together The reason that the two jaundices could be differentiated rather than be assumed as recurrences was the fact that other men in the division were having the epidemic disease Under those circum stances one can usually differentiate but in many instances recur rence is a difficult thing to rule out

Smetana On histologic evidence with repeated biopsies up to about a dozen we sometimes find that nonfatal infectious hepatitis can last for several years It is a smoldering affair producing very small foci of necrosis and the question comes up where is the virus and how does it survive? Is it a symbiosis between the liver cells and the virus? Does it remain inactive if the patient is well? Does it become active again when the patient is subjected to

changes? Is there any clinical indication that this is the case, or is this all wrong?

Best Do the lesions prove that the virus has persisted there?

Smetana The lesions appear acute and active

Best Is the virus there?

Smetana I don't know, but, clinically, the patient has hepatitis, that is, the function tests are in favor of this diagnosis and he is considered to be a case of hepatitis

Best You would really have to recover the virus from the specimen, wouldn't you, to prove it?

Smetana To be absolutely sure yes But the clinician is satisfied with the diagnosis and pathologically we find it compatible with persistent hepatitis

REFERENCES

1 HAWLEY, W L McFARLAN A M and STEIGMAN A J Hepatitis following injection of mumps convalescent plasma III Clinical and laboratory study *Lancet* 1, *818 (1944)*

2 HOAGLAND C L and SHANK R E Infectious hepatitis A review of 200 cases *J A M A* 130, *615 (1946)*

3 ZIMMERMAN H J, *et al* Infectious hepatitis clinical and laboratory features of 295 cases *Am J Med Sc* 213, *395 (1947)*

4 FOX J P *et al* Observations on the occurrence of icterus in Brazil following vaccination against yellow fever *Am J Hyg* 36 *68 (1942)*

5 CULLINAN, E R The epidemiology of jaundice *Proc Roy Soc Med* 32, *933 (1939)*

6 FINDLAY G M and MACCALLUM, F O Note on acute hepatitis and yellow fever immunization *Tr Roy Soc Trop Med & Hyg* 31, *297 (1937)*

7 THEILER A Acute liver atrophy and parenchymatous hepatitis in horses *Reports of the Director of Veterinary Research Union of South Africa Dept of Agriculture* 5-6, *7 (1918)*

8 MACCALLUM F O, and MILES J A R A transmissible disease in rats inoculated with material from cases of infective hepatitis *Lancet* 1, *3 (1946)*

9 LEFTWICH W B and MIRICK G S The effect of diet on the susceptibility of the mouse to pneumonia virus of mice (PVM) I Influence of pyridoxine in period after the inoculation of virus *J Exper Med* 89, *155 (1949)*

10 MIRICK G S and LEFTWICH W B The effect of diet on the susceptibility of the mouse to pneumonia virus of mice (PVM) II Influence of pyridoxine administered in the period before as well as after the inoculation of virus *J Exper Med* 89, *175 (1949)*

11 SMITH, J M, MURPHY, J S, and MIRICK, G S The effect of adrenal hormones on infection of mice with pneumonia virus of mice (PVM) *Proc Soc Exper Biol & Med* 76, 505 (1951)

✓ 12 MELCHER, G W, JR, BLUNT, J W, and RAGAN, C Occurrence of hepatitis in cortisone treated rats following inoculation with human infectious hepatitis serum Proceedings of the Forty fourth Annual Meeting of the American Society for Clinical Investigation *J Clin Investigation* 31, 649 (1952)

13 MIRICK, G S, *et al* The enhancing effect of urethane on the severity of infection with pneumonia virus of mice (PVM) *J Exper Med* 95, 147 (1952)

14 JORDAN, J, and MIRICK, G S Hepatitis in mice, of presumed viral origin (a preliminary report) *Bull Johns Hopkins Hosp* 89, 326 (1951)

15 LUCKÉ, B The pathology of fatal epidemic hepatitis *Am J Path* 20, 471 (1944)

16 LUCKÉ, B, and MALLORY, T B The fulminant form of epidemic hepatitis *Am J Path* 22, 867 (1946)

✓ 17 DOLJANSKI L, and ROSIN, A Studies on the early changes in the livers of rats treated with various toxic agents, with especial reference to the vascular lesions *Am J Path* 20, 945 (1944)

18 MALLORY, T B The pathology of epidemic hepatitis *J A M A* 134, 655 (1947)

19 PERRIN, T L Spontaneous and experimental encephalitozoon infection in laboratory animals *Arch Path* 36, 559 (1943)

20 KLEIN, E, KURNICK, N B, and KLEIN, G The effect of storage on the nucleic acid content and virulence of mouse ascites tumor *Exper Cell Res* 1, 127 (1950)

21 OLITSKY, P K, and CASALS, J Certain affections of the liver that arise spontaneously in so-called normal stock albino mice *Proc Soc Exper Biol & Med* 60, 48 (1945)

✓ 22 GLEDHILL A W, and ANDREWES, C H A hepatitis virus of mice *Brit J Exper Path* 32, 559 (1951)

23 TRAUB E An epidemic in a mouse colony due to the virus of acute lymphocytic choriomeningitis *J Exper Med* 63, 533 (1936)

24 GYORGY, P, STOKES, J JR, and GOLDBLATT, H Antimicrobial agents in the prevention of experimental dietary injury of the liver *Tr Assn Am Physicians (Philadelphia)* 64, 289 (1951)

25 LEIBOWITZ S Heterophil antibody in normal adults and in patients with virus hepatitis *Am J Clin Path* 21, 201 (1951)

26 HAVENS W P JR, GAMBESCIA J M, and KNOWLTON, M Results of heterophil antibody agglutination and Kahn tests in patients with viral hepatitis *Proc Soc Exper Biol & Med* 67, 437 (1948)

27

tion 23, 417 (1944)

28 ALLEN, J G, *et al* Homologous serum jaundice and its relation to methods of plasma storage *J A M A* 144, *1069 (1950)*
29 RATNOFF, O D, and MIRICK, G S Acute hepatitis in general hospital practice *Bull Johns Hopkins Hosp* 85, *299 (1949)*
30 KAHN, A, JR, ARMSTRONG, H M, and EUBANKS, R M Virus hepatitis in laboratory workers *Am J Clin Path* 22, *56 (1952)*
31 VOLWILER, W, JONES, C M, and MALLORY, T B Criteria for the measurement of results of treatment in fatty cirrhosis *Gastroenterology* 11, *164 (1948)*
32 GAULD, R L Epidemiological field studies of infectious hepatitis in the Mediterranean Theatre of Operations *Am J Hyg* 43, *248 (1946)*

ENZYME ACTIVITY IN HUMAN LIVER*

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WHEN PROFESSOR BEST invited me to take part in this discussion, I was hesitant because the work I have been doing on enzyme activity in the human liver is very much in a preliminary stage with no conclusive results yet But, of course, that is just the stage when I need the criticism and advice and help which I expect to get from this group There is a particular difficulty in work of this kind in which one is trying to apply biochemical methods to a human problem The results are very crude when judged by the standards of *the enzymologists, who, as someone said to me the other day*, walk with the gods, and, on the other hand, they are not yet of any practical value to the clinicians

My interest is in so-called tropical nutritional liver disease or the group of such diseases This work was begun in the Gambia British West Africa, in Professor Platt's unit (1,2), and continued in Jamaica I should like to record my gratitude to Professor Kenneth Hill, who attended this Conference last year, for allowing me to use material from his cases, and particularly to Dr K Rhodes, who has done all the clinical work in Jamaica and given me every *possible help*

You will appreciate that in tropical countries there are difficult conditions so that much has not been done which ought to have been done, but the more limiting factors the more necessary it is, I think to choose the right things to do Here again I am looking for help from this group

My remarks come under three headings the objectives of the work the methods and the results the last being very thin As regards objectives I started with the obvious and, no doubt, naive

* This work was supported by the Medical Research Council London It was begun in the Council's Field Research Station Fajara Gambia British West Africa under the direction of Professor B S Platt to whom I am deeply indebted for stimulus and ideas I am grateful also to Dr J H Walters for clinical collaboration. The work was continued in the Department of Physiology University College of the West Indies in collaboration with Professor K R Hill and Dr Katerina Rhodes.

idea that if any of the forms of liver damage that are so common in the tropics are really caused by malnutrition, as many of us believe, then the structural lesion ought to be preceded by a metabolic change, a biochemical lesion In fact I admit that with the evidence available, the search for such a lesion is at the moment a hit or miss affair, and the approach is perhaps rather unscientific

In deciding to study the enzymes in the liver, I was guided by three thoughts first that it is likely to be more fruitful to measure activities or rates of change in the tissue itself rather than concentrations or levels of metabolites in blood or urine For that purpose, there are two main methods studies with tracers and measurements of enzyme activity I think I am right in saying that at present, odd though it may seem, isotope methods are not sensitive enough for use either *in vivo* or *in vitro*, with the very small amounts of tissue that we can get by biopsy in the human subject I may be wrong on this But, on the other hand, thanks to the techniques developed by Linderstrøm Lang and Holter (3 4,5 6), these amounts of tissue are more than adequate for the measurement of many enzyme systems

The second point is that broadly speaking in the tropics, where liver damage is very common, the diet is low in protein or at least in animal protein We know from the work of Addis (7), Koster-litz (8), and others, that under such conditions much cytoplasmic protein is lost from the liver In recent years, a number of workers, such as Miller (9), Rosenthal (10), Schultz (11), Williams (12 13), Bargoni (14), and others have shown that with starvation or diets low in protein or lacking single amino acids, some enzymes are greatly reduced in the liver, others are selectively preserved Admittedly at the moment there does not seem to be very much rhyme or reason about which enzymes are lost and which remain in full activity Among the ones found to be reduced, I might mention xanthine oxidase choline oxidase, catalase, arginase, rhodanese and cathepsin That list does not at the moment make very much sense but at the beginning of this work there did seem to be good reason to suppose that we might find changes in the pattern of enzyme activity in man as a result of malnutrition

Lastly the low protein intake of these people is often accompanied by a low intake of B complex vitamins, therefore, enzyme activity in many cases might be limited by a shortage of co factors With appropriate conditions of assay, one might be able to detect that Again there is experimental evidence for such a thing happening I am sure everyone is familiar with the finding by Elvehjem

and his co-workers (15,16) that in the rat riboflavin deficiency causes a decrease in xanthine oxidase and d-amino acid oxidase activity in the liver, both of these, of course, being flavoprotein enzymes

Those were the ideas I had in mind I hoped that if a decrease in certain enzymes was found, I might get information of two kinds first, that the nature of the enzyme lost might give some indication of the nature of the deficiency (I mean, for instance, that if it were found that all the enzymes affected were sulfhydryl enzymes, it might point to S amino acid deficiency as the limiting factor in the diet), secondly, if I knew the function of the enzymes tested, it might give some clue as to how the deficiency affects the metabolism of the body and tissues as a whole

That is looking into the future, the results have not yet fulfilled *either of these expectations One difficulty is that except for a few* isolated observations on cytochrome oxidase and succinoxidase *mainly in connection with cancer, there is as far as I can find, no* information in the literature about enzymes in the human liver, and so it is first necessary to get some idea of the pattern in general in man as compared with other animals

I should like to say a word about the clinical material I work entirely with children, and mostly with babies, 1 to 3 years old The reason for this is, first, that they show liver changes in the most acute form (I suppose because the growing organism is more vulnerable to a deficiency), secondly, I want to get them if possible before there are advanced changes Moreover, in older children or adults it may be hard to find a completely normal liver

There are two difficulties The first is that we do not have any true controls In the tropics particularly in Africa where malaria *is so common it is hard to say that any child is normal* I have tried to get around this by arranging the material so that each case as far as possible is its own control, with two biopsies, one on admission to hospital and the other after a month or so of treatment

The second difficulty is that, obviously, in order to draw any sort of conclusions we have to try to get a fairly homogeneous clinical series just as in a laboratory experiment That is not easy In Africa I used to go around the bush villages and choose cases there, primarily babies who were just *undernourished* and bring them into the hospital We tried to find out whether what might be called *simple malnutrition* was in any way affecting the liver Even these cases however were complicated because all the babies had a

heavy malarial infection In Jamaica, I have been working on the cases that come to Dr Rhodes' liver clinic, and they are often in a rather more advanced stage, with clinical evidence of liver damage, such as enlargement and sometimes ascites, a rather mixed group of cases But I thought that for the time being it might not be a bad thing just to take what came and work on it and see if anything interesting turned up So at the end when it comes to the results, I must apologize that I cannot produce any sort of averages of groups which can be analyzed statistically, but can only give examples of individual cases

The methods in this work have been determined by the amount of tissue available, which isn't very much I am content if I can get 2 or 3 mg, because we can't go as deep into these babies as in a biopsy on an adult And since the pathologists need a fairly large

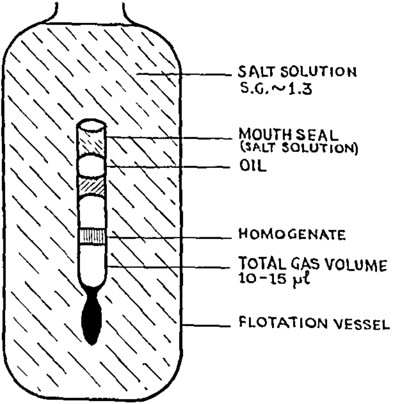


FIGURE 5 Diver in flotation vessel

sample to be representative, there is not very much left for me

I have tried to scale all my methods so as to operate at a level of about 1 mg wet weight of tissue I have used two main methods of measurement gasometric and colorimetric The gasometric method is the Cartesian diver technique of Linderstrøm Lang and Holter The diver is a capillary closed at one end, floating in a salt solution of density about 1 3 (Figure 5) The diver has to be made so that its gas volume matches its weight, so that it will just about float The divers are run in a series Figure 6 shows three flotation vessels with divers in, connected with a manometer and a pressure regulator To get a reading of any kind, all that has to be done to adjust the pressure, either positive or negative, to get the diver just floating in the middle of its vessel and, as far as possible, stationary It is an unstable equilibrium, it is never absolutely stationary The diver is observed through a horizontal microscope, and the pressure read on the manometer If, for instance, the diver contains something which is taking up oxygen, its gas volume will decrease, and it will require a more and more negative pressure to bring it up to its equilibrium position

I have tested this method fairly carefully with simple systems, such as evolution of nitrogen and carbon dioxide, it is accurate to

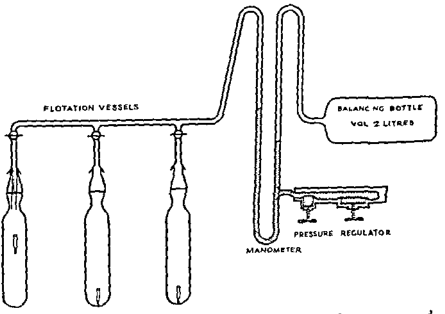


FIGURE 6 Diver apparatus showing flotation vessels manifold manometer and pressure regulator

1 per cent with evolutions of gas of the order of 0 1 or 0 2 cu mm However, I don't want you to think that we regard this as particularly sensitive, because some spectrophotometric techniques are even more so The other day Dr Baird Hastings showed me some details of a new method, worked out by Dr Scholander, which is 5000 times as sensitive as the diver, but not, I think, as versatile

The diver method is versatile, it can be used with different gas fillings, not only with air, but with oxygen, or carbon dioxide, or nitrogen Reagents can also be mixed in the diver, just as in the Warburg The diver is coated on the inside with silicon, the reagents are placed in drops on the walls and mixed with the homogenate by applying a positive or negative pressure (Figure 7)

The other method I have used is a colorimetric one, also based on an instrument developed by Dr Holter* The solution to be

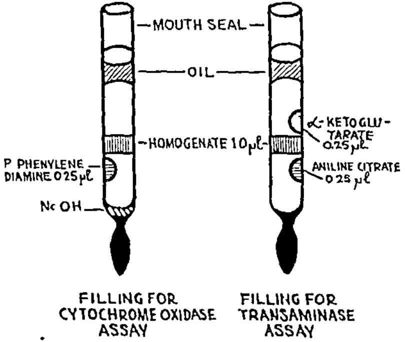


FIGURE 7 Arrangement of reagents in divers for cytochrome oxidase and transaminase assays.

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* This instrument is essentially similar to that devised by Dr Pollister for measuring light absorption in tissue sections (17)

measured goes into a microcuvette (Figure 8), which is simply a microscope slide with a short length of thick walled capillary sealed to it, of about 1 mm internal diameter The top is closed with a coverslip, so that the total volume of solution is anything from 8 to 20 or 30 cu mm, according to the diameter of the

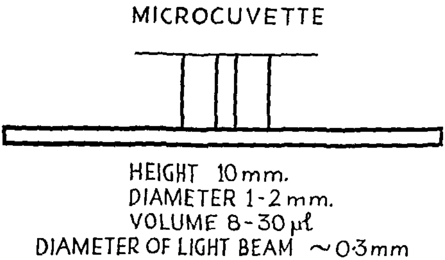


FIGURE 8 Microcuvette

capillary The cuvette goes on the stage of a microscope, which I use *in the horizontal position A tiny light beam, about* 0 2 mm diameter comes up through the cuvette into the microscope (Figure 9) There is a prism and a side tube for centering and an arrangement for holding filters The light goes onto a photomultiplier tube fixed over the eyepiece, and readings are made on a galvanometer In principle, it is an ordinary colorimeter but with cuvettes that will take about 10 cu mm

What I do with these biopsy specimens is usually to divide them into two parts Each weighs about 1 or 1 5 micrograms They are weighed on a quartz fiber balance to about 2 micrograms One part is homogenized in 0 3 molar sucrose, *and used for the enzyme* measurements in the diver and the other part is dried over phosphorus pentoxide It is then extracted with ether, and the fat content measured by weighing again, by difference That, of course, does not give a complete total extraction of the lipoids but I think it is good enough for telling whether there is much fat in those livers

The defatted liver is extracted with hot trichloracetic acid I measure the desoxyribose nucleic acid content of the extract by the

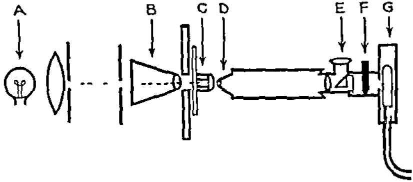


FIGURE 9 Apparatus for colorimetric measurements using microcuvette A light source B substage condenser C microcuvette D microscope objective (x10) E side tube with prism F filter G photomultiplier tube

diphenylamine color reaction (18) Another part of the extract is diluted and the total U V absorption measured at 260 millimicrons That gives the total nucleic acids By subtracting the DNA of course using the correct absorption factor for DNA and RNA one can get the RNA by the difference On a specimen therefore I know the dry weight the fat content the DNA and RNA then the residue after trichloracetic acid extraction can be used for nitrogen measurements which should give the protein nitrogen That is more or less the routine at the moment

There are of course a number of technical points which I am sure will bring criticism The first thing that will be asked about is the reproducibility of the results and what they mean

The diver method itself is accurate to about 1 per cent But using homogenates I can t achieve anything better than 5 per cent reproducibility as a routine and often the error is greater than that You will realize that in trying to do three or four enzyme systems one has to work fast and I do find that that to some extent impairs my accuracy Everything is done in duplicate but I would not like when considering results to put much weight on differences of less than 20 per cent or so The error is partly personal I have been faced with the problem that the more measurements I do the harder it is to do them accurately It is a choice between precision and scope At the moment I have chosen to make the scope as wide as I can and later I shall concentrate on precision on points that seem interesting

The question of sampling error is important This 2 mg is only about 1 200 000 of the weight of the whole liver and obviously

it may not be representative That is a difficult point to overcome The only way I have been able to do it is to make separate homogenates Sometimes when the biopsy is not too successful we go in two or three times and take out several small fragments The homogenates are made either from those or by cutting up one larger fragment Of course those admittedly are not going to be from regions of the liver very far apart Although I have considered it I never thought that I would be justified in doing separate biopsies say on the right and left lobe In Table VI there are some figures that compare the results on separate homogenates from the same case

TABLE VI

Comparison of the Cholinesterase Activity of Different Homogenates

Homogenate 1	Homogenate 2
27 0	25 4
30 9	31 5
16 6	17 8
31 3	29 8
31 3	3[illegible] 1
23 8	20 7
35 3	45 7
10 9	13 6
28 2	27 8

Reprinted by permission from Waterlow J C Observations on the activity of some enzymes in the human liver West Ind Med J 1 41 (1951)

For cholinesterase activity except for one result the agreement is reasonable There is no gross discrepancy Table VII shows various enzymes done on duplicate homogenates I put down all the figures I had to be fair The first figures were from a liver where histologically there were fibrous bands and I could not get a very good homogenate I found the fibrous tissue sticking to the pestle As I said at the beginning I usually try to get these livers before there is much fibrosis Except for one rather bad result the agreement here is again reasonable Therefore I feel that provided we are dealing with early cases and cases in which the

TABLE VII

Activity of Duplicate Homogenates

Case	Enzyme	Homog 1	Homog 2	
Wilson	Cytochrome Oxidase	6 1	3 8	Fibrous Liver
Hunter	Cytochrome Oxidase	11 4	10 9	
	Cholinesterase	5 5	5 2	
Sinclair	Cytochrome Oxidase	7 8	7 6	
	Cholinesterase	8 4	7 2	
	Lactic Dehydrogenase	9 9	9 65	
Linton	Lactic Dehydrogenase	16 7	23 4	
Ellis	Lactic Dehydrogenase	7 0	6 1	

pathologic lesion is known to be diffuse, we can assume that this little bit of tissue is reasonably representative

The next difficulty is the basis of representation In most of the work in animals on enzyme activity in relation to nutrition, the authors express their results per whole liver, or per hundred grams of animal Obviously, that can't be done in this case because of not knowing the volume or weight of the liver The obvious base of representation dry weight, must clearly be corrected for fat and for glycogen Glycogen is, I think, more abundant in the human liver than in most animals From the figures of Holmes and Trowell (19), who are the only people I know of who have estimated glycogen in human biopsy, it may amount to as much as 20 per cent of the dry weight The dry weight, even after correction for fat and glycogen may be misleading because, as Kosterlitz and others have shown in malnutrition or protein deficiency the cytoplasm is reduced the cells become smaller, and there are more cells per unit weight decrease of activity per cell might then be masked by this increase in number of cells per milligram

I tried to get around this by measurement of DNA, but that is not a perfect solution We know that the DNA content of each diploid nucleus is supposed to be constant, but in the liver a large and variable number of nuclei are not diploid, tetraploid, or even octoploid Polyploidy is particularly common in infants and especially in a damaged liver where microscopic evidence of regeneration can be seen

I don't know whether the nucleocytoplasmic ratio remains constant in these large cells Perhaps somebody can help me with that If it remained normal, it would not matter very much

Another source of error is the fact that there are, after all many cells in the liver which are not hepatic cells I think that people *working with rat livers, particularly the biochemists* do tend to assume that they have a homogeneous tissue That may be true for the rat but from microscopic counts I have made in the most normal human specimens I could find, that is, African specimens the nonparenchymatous elements, Kupffer cells and probably some leukocytes in the portal tracts may amount to as much as one third of all the cells present Very often, they may be more than that because in human livers, very frequently cellular infiltrations of no particular significance, often quite fleeting, are seen And as many of those cells, particularly, I think, lymphocytes, contain a lot of DNA compared with their total volume, they could badly upset results which are based on DNA content I have tried to choose the least erroneous method, and for the moment I express my results per milligram protein nitrogen, that is to say, the nitrogen measured in the residue after trichloracetic acid extraction I think that *should* be reasonably sound except in cases where the tissue is diluted with a lot of inert protein, like collagen but as I said before, I try to avoid that kind of case anyway

Best Shall we have a little debate on that now? Dr Stetten what do you think is the best way of expressing results on liver tissue?

Stetten I don t think there is any one way, Dr Best Any basis of reference suffers from intrinsic errors It depends largely on what the point is that one is trying to make It is generally held that most of the proteins of the liver are not soluble in fat For this reason I would certainly prefer the defatted liver weight to the total liver weight particularly if the fat content varies I think it depends entirely upon what the writer is trying to emphasize, and I personally feel that is one of the things that should be left to the writer's discretion

Fremont Smith Provided he specifies

Stetten Yes it should always be made perfectly clear It is also generally possible so to present data that if the reader chooses to refer to some other standard of reference he can do so

Best I have felt that in some papers the authors just eliminate the fat because there is too much and in others they say, "Lets eliminate the glycogen" I say why not eliminate the protein too?

Knisely Recently in the *Scientific American* there was an article by a good biophysicist who said that we have never had and do not today have a competent method of measuring the volume of one cell

Waterlow Linderstrøm Lang and his co workers in Copenhagen have developed a method of measuring specific gravity of single cells by reduced weight This would give the volume if the true weight were known

Elias May I ask Dr Waterlow how do you determine the polyploidy of nuclei?

Waterlow I have not determined it myself

Elias How could it be be done?

Waterlow From what I have read it has been determined in two ways first of all by measuring the diameter and hence the volume of nuclei and finding that there is a distribution curve with volumes in the ratio of 2 4 and 8 Whether the volume really tells the number of chromosomes of course I dont know Harrison in McCances laboratory has recently made an estimate of the average degree of polyploidy by comparing the DNA content per cell in liver and kidney (20) I am sure Dr Pollister can answer this question much better than I

Pollister We have only a few data on biopsied livers in which we have estimated it by the Feulgen reaction These were adult livers two cases so far

Best What was the reaction?

Pollister The reaction was that for desoxypentose using a micro photometric apparatus and taking at random the individual nuclei There seems to be a rather small percentage of polyploids in these adults it is quite small in comparison with rodents in which tetra ploids and octoploids make up more than half of the parenchymal nuclei In the human material tetraploids were very few I think we found only one octoploid in some two hundred nuclei

I was interested to hear that you thought the incidence of poly ploidy might be exceptionally high in these children if that is so it is just the reverse of the situation in rodents in which the per centage of polyploid nuclei steadily increases with age

Waterlow I dont know whether it is a question of age or of injury but certainly in these children there are very many more large nuclei and binuclear cells than one expects in an ordinary liver and than I find in European livers

Pollister The time of high polyploidy in man may well be at a different time of life than in the rat The size does not correlate with DNA content as strictly as one might perhaps expect For example, nuclei are found with diploid DNA amount which are as large as the average tetraploid These swollen diploids and tetraploids can always be distinguished at a glance for the latter are both larger and darker

Waterlow Yes

Pollister Thick sections must be cut, so that the whole nucleus is in the section

Elias Dr Pollister's comment that in rodents there is more polyploidy than in man is easily explained I don't know how it applies to pathology but, as I hope to show in my presentation, the size volume, shape, and number of nuclei in liver cells in normal livers are dependent on their location in the wallwork of the liver plates, and it is a geometrical necessity for each individual liver cell to be so shaped and to have the volume which it has The volume depends on the number of nuclei or the diploidy or polyploidy, whatever the reason may be

It so happens that those cells which are located in the corners where three liver plates meet are the largest, and they usually have two or several nuclei, or one large polyploid nucleus, and the number of corners per area is much larger in the rodent liver than in the human liver, because that is of the tubulosinusoidal type whereas the human liver is of the sacculosinusoidal type

Shorr May I ask Dr Waterlow about the respiration of the fat in these fatty livers, that is, does the fat in the fatty liver have an appreciable oxygen uptake? I had some experience with fatty livers in rats and found that the average oxygen consumption in these fatty livers, using wet weight, was just about the same as that of a normal liver Therefore, either the cells were respiring at a greatly increased rate or the fat of the liver itself had a distinct oxygen uptake

Best The fat might be a substrate

Shorr Fatty tissue elsewhere has an appreciable oxygen uptake

Gyorgy It could be substrate

Best That is quite a question I don t think we can ask Dr Waterlow

Waterlow I do not have any means of examining the respiration of the fat itself

Popper We might consider the enzyme concentration of the liver an indication of its metabolic activity As Koch Weser and

Farber (21) have shown in our laboratory in the acute fatty liver produced by ethionine administration to female rats the enzyme content is not altered if the initial body weight of the animal is taken as reference That means in the acute fatty liver without necrosis (in contrast to the fatty liver with necrosis) it is not altered from the normal The enzymes studied were the alkaline phos phatase esterase and succinic acid dehydrogenase

Lagerstedt I agree with Dr Popper especially if the localization of activity within the liver cell is considered As far as I know it has not definitely been settled which of the particular bodies are responsible for different enzymatic activities which can be recog nized directly in the liver cell

Smetana One of the histologic characteristics of liver disease is the variance in size and shape of liver cells and nuclei Do you take that into consideration?

Waterlow That is what upsets my measurement based on desoxy nucleic acid I am very much aware of that

Smetana Can you correct for that?

Waterlow I don t think I can Really what I am trying to get is the active protoplasmic mass I should like of course as well to have some indication of the activity per cell if I knew the number of nuclei

Kark What happens when you relate your results to the body weight of the child? Have you done that?

Waterlow I have never done that no In a way I can t do that because I have a given amount of tissue and I don t know how big the liver is

Kark That doesn t matter I am talking about the entire nutri tion of the child

Waterlow You mean I could measure oxygen uptake which is microliters per milligram and then plot that against body weight?

Kark Yes

Waterlow Yes if I had a big enough series That ought to come out in the results that I shall show you but I don t think it does

Best Dr Waterlow will you resume the account of your coura geous attack on a very difficult problem

Waterlow There is just one more point on the technical side that I want to make which is intended really to excuse myself for not having very many results and that is the fact as I think all enzyme workers in this field always point out that one has to be extremely careful about conditions of assay Even with every pre caution one can t really be certain that one is assaying the thing

Pollister The time of high polyploidy in man may well be at a different time of life than in the rat The size does not correlate with DNA content as strictly as one might perhaps expect For example, nuclei are found with diploid DNA amount which are as large as the average tetraploid These swollen diploids and tetraploids can always be distinguished at a glance, for the latter are both larger and darker

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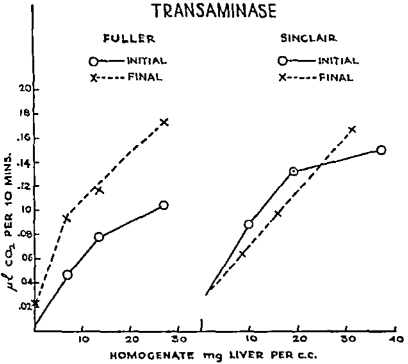


FIGURE 10 Transaminase activity of human liver at different enzyme concentrations

Lagerstedt Dr Waterlow, there is one experience I have had in homogenizing liver cells using an all glass homogenizer I made it in the original way in a 0 88 M sucrose solution I was primarily interested in getting an account of the number of cytoplasmic components from that liver Now some nuclei might be destroyed too and thus there will be nuclear material in the homogenate So I made a counting of the number of free nuclei after prolonged homogenization I found that after a certain time there is a decrease in the number of free nuclei So I drew the conclusion that that homogenization apparatus must be used with an awareness of this danger viz that one is really also crushing nuclei

Waterlow I am aware that obviously the conditions of homogenization might easily affect the results I never really had time to investigate that, I just try to keep the conditions standardized I always homogenize for half a minute with the tissue in ice That is the only control I have done on that To tell you the truth I have

one thinks one is, and all the conditions ought to be worked out for the particular tissue, factors like pH optimum, concentration of reagents, and, above all, stability of enzyme I don't think it is justifiable to assume that what is found in the rat, for instance, is going to hold for man It takes a long time to test all these points and I have not always succeeded in doing it thoroughly

Finally, there is one thing that is essential for any assay of enzyme activity, and that is that the activity should vary linearly with the amount of tissue used With most of the enzymes I have worked on, that is the case, except for one, transaminase

In Figure 10, the amount of tissue expressed as milligrams of liver per milliliter of homogenate is shown below Actually, of course, the amount of tissue is of the order of a fiftieth of a milligram in one microliter It will be seen that the carbon dioxide output does not go completely linearly with the amount of tissue The curve falls off with increasing amounts of tissue The two curves in each case are activities in initial and final biopsies I have the impression that the falling off is more severe in the initial biopsy which to me suggests possibly a deficiency of some other factor, a co factor I don t know if that is a correct deduction, but I have obtained some pyridoxal phosphate and I hope to try it out

Shorr The tissue volume is the same?

Waterlow The volume of fluid is the same in each measurement that is, one cubic millimeter I started with a concentrated homogenate and diluted it by two, and then diluted that by two again

Shorr Isn't that characteristic of diluted homogenates?

Waterlow You mean there might be a dilution effect?

Shorr Yes

Waterlow You see the activity is all right at low concentrations It falls off with the higher amounts of tissue A dilution effect would be the other way around

Pollister Could you tell us how completely the cells have been disrupted?

Waterlow I have not examined them all microscopically, but when I have I have usually found just one or two intact cells when I made a smear They are mostly broken up

Grafflin How do you homogenize?

Waterlow I homogenize in a little glass tube, about 2 mm in diameter and use a glass pestle

Kark You couldn't freeze first?

Waterlow Freezing is known to destroy a great many enzymes not all

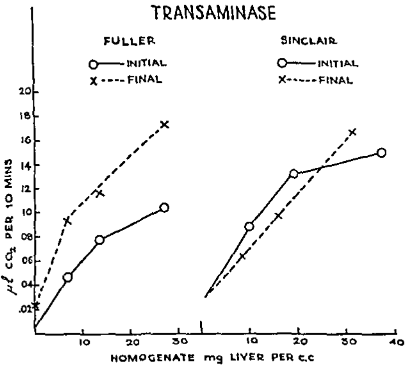


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rather taken the view that what is good enough for Dr Potter and his colleagues is good enough for me

Grafflin Are you using a Potter homogenizer?

Waterlow It is much smaller than a Potter

Grafflin Are you using ground glass surfaces, without projecting beads on the pestle?

Waterlow Yes I can only say that mostly when I have checked it, the particles in the homogenate are very small I suppose I have many crushed mitochondria, and so on

Coming to the results, the first point is perhaps the choice of enzymes measured Obviously, no more than a limited number can be measured in one bit of tissue, not so much because of the amount of tissue but because of time, as the enzymes are unstable My choice has been based on two criteria first, that the enzymes should be physiologically interesting and, second that the methods of assay should be accurate

TABLE VIII

Comparison of Human and Rat Liver

	Man	Rat
Cytochrome Oxidase $-QO_2/N$	125	950
Lactic Dehydrogenase $-QO_2/N$	230	320
Cholinesterase QCO_2/N	95	140*
Transaminase QCO_2/N	2350	340
Succinoxidase $-QO_2/N$	15	600†

* Female rats
† *Figure from the literature*

Table VIII shows that with some enzymes, there are considerable differences in activity between rat and man The most striking difference is in the succinoxidase, which has been so much worked on in the rat and which I have hardly been able to assay in man Cytochrome oxidase is much less active in man than in rat, lactic dehydrogenase about the same, cholinesterase about the same In the case of transaminase, the opposite situation holds it is about *eight times as active in man*

These are averages from my small series Most enzymes I had expected to be considerably less active because of the effect of

body size, and I was rather surprised when this fact came out about the transaminase I don't know what it may mean, but obviously it is not much good trying to find differences in enzyme activity if the activity is so low that it can hardly be measured, so recently I have concentrated on the first four in that group* and have not done measurements of succinoxidase The values (Table VIII) are expressed as QO_2/N, oxygen uptake per milligram protein nitrogen Some of the earlier work is expressed as QO_2 per milligram fat free dry weight, and the later work I have expressed as per milligram protein nitrogen, for reasons I gave earlier

Cholinesterase was one of the first enzymes I attacked McCance and his colleagues in Germany (22) showed that plasma cholinesterase was low in repatriated prisoners of war and other undernourished people Figure 11 is a case from Africa of a baby about 18 months old, with edema It can be seen from the weight curve that as it was treated with a high protein diet, the edema disappeared, and the weight began to rise rapidly with good feeding Plasma protein levels were reduced initially, but rose to 7 per cent with treatment Plasma cholinesterase was much more severely reduced than plasma protein The normal plasma esterase level by our method is about 70 units In this case, it started at about 10 units, so that the reduction was much greater in esterase than in protein This reduction in the plasma was accompanied by a rather low liver esterase, as represented by the blocks in the Figure

* Technique of enzyme assays
For each assay there was used 1 or 1 25 cu mm of homogenate made in 0 3 M sucrose
Figures in parenthesis are final concentrations

Cytochrome oxidase	
substrate p-phenylenediamine	(03 M)
phosphate buffer pH 7 4	(033 M)
cytochrome c	(0001 M)
aluminium chloride	(0004 M)
gas phase air	
Lactic dehydrogenase	
substrate Na lactate pH 8 3	(03 M)
phosphate buffer pH 8 3	(06 M)
sodium cyanide pH 8 3	(05 M)
methylene blue	(04 per cent)
coenzyme I approx	(003 M)
gas phase air	
Cholinesterase	
substrate acetylcholine	(02 M)
sodium bicarbonate	(032 M)
gas phase 95% O_2 5% CO_2	
Transaminase	
sodium α ketoglutarate pH 7 4	(066 M)
" aspartate	(033 M)

(After 10 minutes incubation mixed with 50% aniline citrate to decompose oxalacetate)

rather taken the view that what is good enough for Dr Potter and his colleagues is good enough for me

Grafflin Are you using a Potter homogenizer?

Waterlow It is much smaller than a Potter

Grafflin Are you using ground glass surfaces without projecting beads on the pestle?

Waterlow Yes I can only say that mostly when I have checked it the particles in the homogenate are very small I suppose I have many crushed mitochondria and so on

Coming to the results the first point is perhaps the choice of enzymes measured Obviously no more than a limited number can be measured in one bit of tissue not so much because of the amount of tissue but because of time as the enzymes are unstable My choice has been based on two criteria first that the enzymes should be physiologically interesting and second that the methods of assay should be accurate

TABLE VIII

Comparison of Human and Rat Liver

	Man	Rat
Cytochrome Oxidase —QO /N	125	950
Lactic Dehydrogenase —QO /N	230	370
Cholinesterase QCO /N	95	140
Transaminase QCO /N	2350	340
Succinoxidase —QO /N	15	600†

* Female rats
† Figure from the literature

Table VIII shows that with some enzymes there are considerable differences in activity between rat and man The most striking difference is in the succinoxidase which has been so much worked on in the rat and which I have hardly been able to assay in man Cytochrome oxidase is much less active in man than in rat lactic dehydrogenase about the same cholinesterase about the same In the case of transaminase the opposite situation holds it is about eight times as active in man

These are averages from my small series Most enzymes I had expected to be considerably less active because of the effect of

These show the activity in the actual liver tissue I should emphasize that this is the so called pseudo or non specific cholinesterase

TABLE IX

Enzyme Activity of the Liver in Malnutrition

No	Case	Fat per cent of dry wt	Cholinesterase Liver QCO_2	Cholinesterase Plasma Units†	Cytochrome Oxidase $-QO_2$	Lactic Dehydrogenase $-QO_2$
1	232 I*	34 5	1 0	25 0	13 6	10 5
2	229 I	—	2 6	23 2	9 9	10 3
	229 F*	5 0	6 2	53 2	13 5	5 85
3	208 I	—	4 5	27 8	20 2	—
	208 F	15 6	10 25	58 7	20 1	6 2
4	250 I	20 5	5 15	34 5	—	3 6
	250 F	11 3	7 85	42 9	—	7 8
5	235 I	17 9	6 9	38 8	10 4	7 7
	235 F	12 4	8 4	60 0	17 1	5 6
6	244 I	23 5	3 6	21 25	—	—
	F	14 6	4 1	19 9	—	9 4
7	215	—	2 95	12 5	17 7	—
Mean Normal		12 65	9 4	60 approx	16 3	5 9

* I = initial biopsy F = final biopsy
† unit = 1 μl per ml of plasma per minute
Reprinted by permission from Waterlow J C Observations on the activity of some enzymes in the human liver *West Ind Med J* **1**, 41 (1951)

Table IX shows results in some other African cases I think it is best to disregard the figures for cytochrome oxidase and lactic dehydrogenase I am not satisfied with the methods then used I just want to point out that again there is reduction in liver esterase in the malnourished subjects compared with the level in controls or the levels reached after treatment The results in this Table are expressed on the basis of dry weight not nitrogen

Case 1 is a baby with edema and a small amount of fat in the liver a very low liver esterase and rather low plasma esterase That child died Case 2 is another baby with a low liver esterase coming up towards normal It did not reach normal the baby was discharged prematurely The first five cases in this Table are malnourished babies arranged in order the nutritional state getting better as one reads downwards There is a reduction in liver esterase

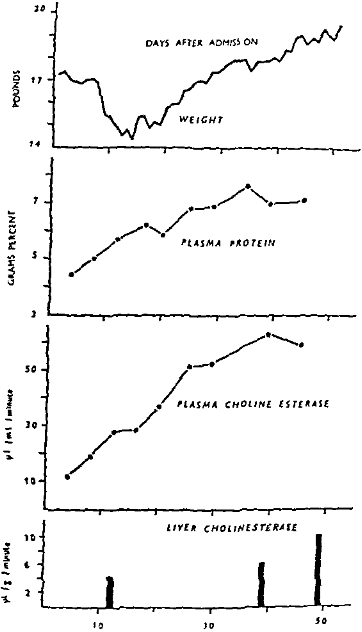


FIGURE 11 Plasma and liver cholinesterase and plasma protein concentration in an African baby Reprinted by permission from Platt B S Report on work of the department of human nutrition *Report of the London School of Hygiene and Tropical Medicine* London 1950 (p 48)

These show the activity in the actual liver tissue I should emphasize that this is the so-called pseudo- or non-specific cholinesterase

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2	229 I	—	2 6	23 2	9 9	10 3
	229 F*	5 0	6 2	53 2	13 5	5 85
3	208 I	—	4 5	27 8	20 2	—
	208 F	15 6	10 25	58 7	20 1	6 2
4	250 I	20 5	5 15	34 5	—	3 6
	250 F	11 3	7 85	42 9	—	7 8
5	235 I	17 9	6 9	38 8	10 4	7 7
	235 F	12 4	8 4	60 0	17 1	5 6
6	244 I	23 5	3 6	21 25	—	—
	F	14 6	4 1	19 9	—	9 4
7	215	—	2 95	12.5	17 7	—
Mean Normal		12 65	9 4	60 approx	16 3	5 9

* I = initial biopsy F = final biopsy
† unit = 1 µl per ml of plasma per minute

Reprinted by permission from Waterlow J C Observations on the activity of some enzymes in the human liver *West Ind Med J* **1**, *41 (1951)*

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roughly parallel with the degree of malnutrition, the level rises with treatment The sixth case *is a child slightly older, 3* years old Pathologically, there seemed to be *an active fibrosis* of the liver with proliferating fibrous tissue, which did not respond *to treatment* The plasma esterase was low at the beginning and end of a month's intensive high protein diet, and liver esterase was also low This reduction in esterase, which seems to be *characteristic of malnutrition* and of liver disease, is not, I think, a very specific thing Case 7 is a child with abdominal tuberculosis and therefore secondary malnutrition, again, there is a low liver and plasma esterase *I think* perhaps I *must* leave for a moment the question of what *this means* Figure 12 shows that there is a relation between the levels of this enzyme in plasma and liver There is a good bit of scatter, but the correlation is statistically significant (Correlation coefficient 0 78 P less than 01)

The next results are from cases in Jamaica I admit they are rather disappointing, I found the reduction in liver esterase two years ago and none of the other enzymes that I have studied since then

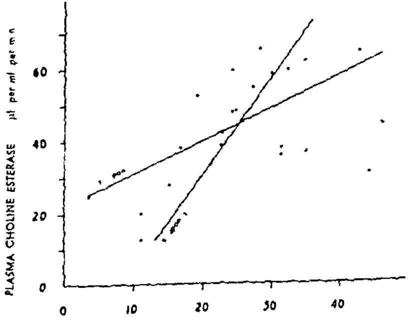


FIGURE 12 The relation between plasma and liver cholinesterase Data from 39 human biopsies Reprinted by permission from Waterlow J C Observations on the activity of some enzymes in the human liver *West Ind Med J* 1, 41 (1951)

ave shown a similar effect Table X and Figure 13 give figures rom a *baby* who had four biopsies The object is to show the kind f variation that there is in the results so that you can judge how ustworthy they are This was a malnourished baby with nothing uch wrong except for weight loss and a slight enlargement of the ver The figures for liver esterase, expressed on the basis of protein itrogen, are fairly constant The low plasma esterase at the time f the first biopsy may, I think, be an artifact, because the reagents ere damaged by a hurricane which had occurred shortly before he cytochrome oxidase is fairly constant until suddenly there is a se in the fourth biopsy That kind of thing happens from time to me double the usual figure is more than can be accounted for ist by error, and I confess I don't know what it means

The figures for lactic dehydrogenase in the first and second biop ies are low because the pH was not optimum I had not discovered



FIGURE 13 Photomicrograph of biopsy specimen of liver from Jamaican infant Hematoxylin and eosin. Normal liver (see Table X)
This and following photomicrographs by courtesy of Dept of Pathology University College of the West Indies

TABLE X

Male, Aged 1½ Years*

Date	Serum Cholin esterase Units	Liver Cholin esterase QCO_2	Cytochrome Oxidase $-QO_2$	Lactic Dehydrogenase $-QO_2$	Transaminase QCO_2	Fat, Percentage of Dry Weight	Protein N, of Non fat Solids	DNA mg/g	RNA mg/g
10/12	0 27	97	149	137*	—	10 6	7 9	14 8	10 2
11/12	0 77	115	135	135*	—	11 5	5 9	17 5	8 5
1/15	0 75	107	124	186	2750	7 7	8 5	15 3	11 4
2/14	0 80	106	266	179	1980	10 0	5 65	10 6	8 7
Average†		95	125	227	2350	10		15 7	10 95

* Moderate malnutrition weight 18 lb, 4 oz Hepatomegaly liver edge 4 cm below costal margin Histology normal parenchyma round cell infiltration of tracts, one area of collagenosis

† As it is difficult to get normal control material for the purpose of comparison the figures from all cases have been averaged to give a rough standard of reference

pH not optimum

that at the time Comparing the third and fourth biopsies there is no significant difference in activity of either lactic dehydrogenase or transaminase

The figures at the bottom of Table X are the average of the results in all my cases I cannot give any figures that are strictly normal but these averages form a rough standard for comparative purposes

In the last column of Table X are figures for ribonucleic acid content measured by the method I described and expressed in milligram per gram fat free dry weight I did not mention this before but you will see that the RNA content is very much lower in the human cases than in the rat In the rat the ratio of RNA to DNA is about 4 1 whereas in these human livers the ratio is often less than 1 My figures for the DNA content are about the same or slightly higher than those found for the rat so that the RNA is very much less Nor does the RNA show any consistent change with improving nutritional status as one would hope This low level of RNA does agree with the fact that at any rate with the staining methods we have used we have been unable to see any of the basophil clumps in the cytoplasm which are characteristic of the rat However I do not want to emphasize this too much at this stage there is a possibility of error Some of the RNA may be being lost particularly as we are working in the tropics and have no cold room This will be investigated

Kark Is that a significant fall in DNA value?

Waterlow Yes In this case the third biopsy showed much lymphocytic infiltration in the portal tracts The last biopsy was completely normal I think these figures show how the DNA standard of reference is upset by infiltrating cells That is my impression That is why the DNA is of rather limited usefulness

Pollister Cell volume might have increased markedly if so the amount of DNA in a given biopsy would surely be considerably less

Waterlow Yes Well I was hoping it would fall with treatment as the cell volume rose

Kark If your cell volume increased that much you should observe an increase in the size of the liver

Waterlow This point was rather puzzling I can t tell you why these cases have hepatomegaly In this case there was not much but many of these children do have livers that I think are abnormally large The cells don t seem big in themselves They don t contain fat There is not much infiltration or anything of that sort

TABLE XI

Case A: Female, Aged 11 Months*

Date	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO	Cyto-chrome Oxidase — QO_2	Lactic Dehydro-genase — QO_2	Trans aminase QCO_2	Fat Per centage of Dry Weight	DNA mg/g
1 2/19	0 53	73	73	197	4170	23 6	18 9
2 3/19	0 78	122	130	—	3000	12 7	12 3

* Weight 12 lb Hepatomegaly liver edge 4 cm below costal margin no edema no ascites With treatment rapid weight gain liver reduced in size Histology slight fatty infiltration cells small

Case B: Female, Aged 11 Months*

Date	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO_2	Cyto-chrome Oxidase — QO_2	Lactic Dehydro genase — QO_2	Trans aminase QCO	Fat, Per centage of Dry Weight	DNA mg/g
1 2/29	0 21	62	198	233	2500	53 2	14 8
2 3/21	0 50	104	177	307	2130		
Average		95	125	227	2350	10	15 7

* Weight 9 lb 12 oz Hepatomegaly liver edge 2 cm below costal margin soft no edema no ascites With treatment rapid weight gain Liver reduced in size Histology severe fatty infiltration

Table XI, Case A is data from a baby rather badly underweight There was a slight enlargement of the liver but nothing much else, no edema or ascites Histologically, there was slight fatty infiltration but no other change A slight reduction in both liver and plasma esterase was observed, and a low cytochrome oxidase, which on treatment came up to normal Whether that is significant, I don t know It occurs in some cases, not in others Transaminase is also at a very high level in this baby As to fat, the normal level of liver fat by my crude method is about 10 to 12 per cent of the dry weight In this case, there was a moderate increase in fat on admission The DNA fell with treatment, just as I had hoped it would and as Dr Pollister suggested It fell between admission and discharge and microscopically one did get the impression that the cells were small and the nuclei crowded in the early biopsy and less so in the later one I have often had that impression before but I very much distrust such impressions because I know what I want to happen and then of course there is the question of artifacts,

fixation, and so on The DNA would be a better index if it were not for cellular infiltration

Case B in Table XI and Figure 14 had a severe fatty liver 50 per cent of the dry weight was fat, equivalent to about 25 per cent of the fresh weight Here again the esterase was low in liver and plasma, but this time a low cytochrome oxidase was not present I am sorry about that So these figures don t mean much The DNA again falls a little between admission and discharge

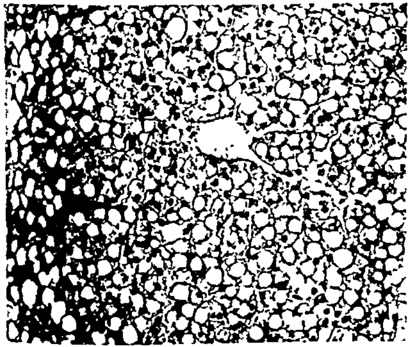


FIGURE 14 Fatty liver (see Table XI Case B)

By the way, in case you wonder what significance the figures for DNA have, I had only two cases with enough tissue to do the whole estimation in duplicate, on separate bits of tissue In those two cases, the duplicate figures in one were 13 4 and 12 3, in the oth 15 8 and 15 5 So I think that a difference of 2 is possibly t taken into account

Hoffbauer Had the fat in the liver m rkedly d time?

TABLE XI

Case A: Female, Aged 11 Months*

Date	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO_2	Cyto-chrome Oxidase $-QO_2$	Lactic Dehydro genase $-QO_2$	Trans aminase QCO	Fat Percentage of Dry Weight	DNA mg/g
1 2/19	0 53	73	73	197	4170	23 6	18 9
2 3/19	0 78	122	130	—	3000	12 7	12 3

* Weight 12 lb Hepatomegaly liver edge 4 cm below costal margin no edema no ascites With treatment rapid weight gain liver reduced in size Histology slight fatty infiltration cells small

Case B: Female, Aged 11 Months*

Date	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO_2	Cyto-chrome Oxidase $-QO_2$	Lactic Dehydro-genase $-QO$	Trans aminase QCO_2	Fat Percentage of Dry Weight	DNA mg/g
1 2/29	0 21	62	193	233	2500	53 2	14 8
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TABLE XII

Case A: Male, Aged 1 Year, 9 Months*

	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO_2	Cyto-chrome Oxidase $-QO_2$	Lactic Dehydro-genase $-QO_2$	Trans aminase QCO_2	Fat Percentage of dry weight	DNA mg/g
1 2/1	0 23	65	94	246	1570	24 7	15 2
2 2/28	0 61	91	142	269	1970	8 9	16 7
Average		95	125	227	2350	10	15 7

*Weight 20 lb Hepatomegaly liver edge 6 cm below costal margin (left lobe) ascites no edema With treatment ascites disappeared and liver reduced in size Histology 1 Patchy centrilobular degeneration Collagenosis (?) 2 Regeneration Hyperplasia of Kupffer cells Extensive nuclear changes in parenchymatous cells

Case B: Male, Aged 2 Years*

	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO_2	Cyto-chrome Oxidase $-QO_2$	Lactic Dehydro-genase $-QO$	Trans aminase QCO_2	Fat Percentage of dry weight	DNA mg/g
1 1/17	0 40	137	235	249	2030	12 6	17 0
2 2/21	0 25	132	127	259	2060	12 5	15 8

*Weight 21 lb Hepatomegaly liver edge 6 cm below costal margin liver hard ascites edema No clinical improvement between biopsies Histology 1) Cellular degeneration and hemorrhage 2) Regeneration and repair

In the initial biopsy, there was marked cellular degeneration and hemorrhage The extraordinary thing was that the enzymes appeared to be extremely active The cytochrome oxidase activity was more than normal and transaminase and lactic dehydrogenase were well up There was no fat and no significant change in DNA

I have shown these slides first to show that the figures are reproducible Secondly, I present them in a way to illustrate my disappointment Being originally very dissatisfied with what I could see with the microscope I had hoped that by this method I could get much more information than the pathologist But actually it turns out that the pathologist can see things when I can t find any chemical changes Whether I shall be able to put that right I don t know

The only consistent abnormality is in the pseudocholinesterase which is troublesome, because we don t know what it does I think there is a possibility that it may have some important metabolic role There is a lot of it in all tissues such as pancreas and gut

Waterlow Yes, the fat had gone That was a normal liver at the end, as far as I could tell

Table XII, Case A, and Figure 15 illustrate a somewhat different case, one with a big liver and ascites In the initial biopsy, there was present the lesion, collagenosis or serous hepatosis, described by Hill (23) I dislike working on material with advanced lesions, but, as I said, I was taking the cases as they came

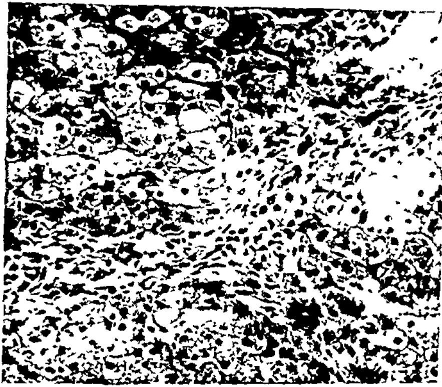


FIGURE 15 Serous hepatosis recovering (see Table XII Case A) Note nuclear changes

The cholinesterase was decreased and the cytochrome oxidase showed a reduction of doubtful significance The transaminase was a good bit lower than the average, but I do not have enough cases to tell whether that has any meaning The second biopsy in this case showed marked nuclear changes large nuclei double nuclei dumbbell shaped nuclei and so on, such as we discussed earlier That may of course account for the slight rise in DNA

The observations in another case rather like the previous one, only worse with ascites and edema are shown in Table XII, Case B

TABLE XII

Case A: Male, Aged 1 Year, 9 Months*

	Serum *Cholin* esterase (Michel)	Liver *Cholin* esterase QCO_2	Cyto chrome Oxidase $-QO_2$	Lactic *Dehydro* genase $-QO$	*Trans* aminase QCO_2	Fat Per *centage of* dry weight	DNA mg/g
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Case B: Male, Aged 2 Years*

	Serum Cholin esterase (Michel)	Liver Cholin esterase QCO	Cyto-chrome Oxidase $-QO_2$	Lactic Dehydro-genase $-QO_2$	Trans aminase QCO	Fat Per centage of dry weight	DNA mg/g
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The only consistent abnormality is in the pseudocholinesterase, which is troublesome, because we don t know what it does I think there is a possibility that it may have some important metabolic role There is a lot of it in all tissues such as pancreas and gut,

which synthesize proteins As you know, proteinases and esterases are probably interchangeable, and if one follows the view of Professor Fruton (24), proteinases can work backward and, in fact, are the only enzymes that are known to work backward in synthesizing peptide bonds One could make a hypothesis that this cholinesterase does play a part in protein synthesis, in which case its reduction would be interesting

Kark In our hands, there seemed to be a direct relationship to body mass, that is, the rate of rise of cholinesterase in malnutrition seemed to bear a direct relationship to the total tissue mass Also

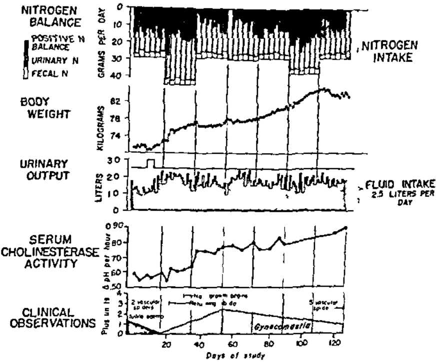


FIGURE 16 The effects of 3 months therapy with a diet low in sodium (33 mEq per day) high in protein (from 181 to 306 Gm of protein per day) and calories together with a fixed fluid intake (2 5 liters per day) on the nitrogen balance body weight urinary output and serum cholinesterase in patient A M ill with cirrhosis and tissue depletion who developed refeeding gynecomastia during recovery and remasculinization Note (a) long continued markedly positive nitrogen balance (b) steady increase in body weight after initial lag (c) long-continued diuresis beginning during the 3rd week of therapy (d) increase of serum cholinesterase ... ilini
especially at th ... s of
zation after 3 ... nter
therapy Repr ... nical
C R Refeed
observations A

there is another bit of evidence to show that it is related to protein metabolism in nephrosis, there are very high serum cholinesterase levels This seems to suggest that the liver is working at a maximum rate to produce cholinesterase and albumin, which of course is being lost via the kidneys

Waterlow As to this idea that the esterase may be active in protein synthesis, I have formed the clinical impression in treating some of these cases that there is a lag before they start getting better One gets the feeling that perhaps the synthetic machinery is itself destroyed, and must be replaced first of all before tissue as a whole can build up That would fit in with these reductions in esterase

I should say that the first biopsy in that last case was done after the patient had been in the hospital for a considerable time, which may be why the esterase activity is high even in the first biopsy

Best There is nothing very definite to relate pseudocholinesterase to protein metabolism

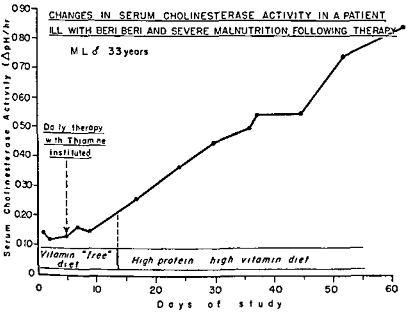


FIGURE 17 Serial observations of serum cholinesterase activity in a patient ill with "wet" beriberi and severe malnutrition before and after treatment with thiamine and a nutritious diet Printed by permission from Vorhaus L J and Kark R M serum cholinesterase in health and disease *Am J Med* (In press)

Kark. I have some data on that Figure 16 gives data from a patient with cirrhosis who was treated with a high protein diet The very positive nitrogen balance can be seen in the area at the top The patient had a high protein intake Below that is body weight, and the parallelism between the body weight and the serum cholinesterase activity can be seen, there being a lag in the serum cholinesterase activity until the body weight starts up

Figure 17 *is a patient with beriberi heart disease, and it shows,* initially, very low serum cholinesterase, which takes a long time to return to normal, about 60 days It did not start to return until the patient was completely cured of his beriberi by thiamine Although the beriberi was cured rapidly, the generalized malnutrition in this patient returned slowly to normal with a good diet, and the cholinesterase rise parallels the return of body mass to normal and bears no relationship to the vitamin deficiency

Best That doesn't seem to me to be related specifically, the one to the other A lot of things might be happening while the body mass is returning to normal This doesn't link protein formation and pseudocholinesterase at all

Kark I am just saying these two things are happening at the same time

Best Yes

Kark That is the only interpretation I can give The rate of formation of protein is speeding up and cholinesterase is being *built at the same time as body protein is being* built

Best I think my reaction is rather protective I have a Ph D student who spent four years trying to find the function of pseudo cholinesterase, without much success If Dr Waterlow comes to Toronto, he can talk to this young lady, who was trained by Dr Mendel who first separated the true and pseudo cholinesterase

Waterlow *Will pseudocholinesterase split a peptide bond?*

Best I don't know

Waterlow I have never tried it, but I gather from the other work on esterases that it might be able to

Watson Mr Chairman, I think the increases in nephrosis that Dr Kark called attention to are really very impressive We have quite a little data now, 15 or 20 cases of either nephrosis or nephrotic states, in which the serum albumin was very low, down around 1 5 Gm or even lower, some down below 1 Gm per 100 ml It is remarkable that the pseudocholinesterase in these people is often in fact, usually, well over one clinical pH unit It is not uncommon

to see it go up to 1 4 or 1 5 far above the normal level in spite of the fact that the albumin is extremely low

In that connection it might be mentioned that McQuarrie and his co workers (25) have shown by means of radioactive methionine that the rate of synthesis of albumin in nephrotic children is greatly increased They are the same ones that have the elevated cholinesterase

Kark We have infused large quantities of albumin into healthy individuals and patients and the serum albumin level goes up and cholinesterase drops down There is a definite parallel between rise in albumin and fall in cholinesterase As soon as the albumin is stopped the cholinesterase goes up again

From our curves we made two interpretations first we felt that albumin and cholinesterase were products of the same synthesis that the liver was very much like any other type of gland for example the pituitary that when the serum level of albumin was raised the liver stopped making albumin and at the same time it stopped making cholinesterase From that one could get the rate of regeneration of cholinesterase we worked out its life span to be about the same as the albumin molecule that is about 28 days

Shorr We studied the rate of regeneration of pseudocholin esterase in cirrhotics who were not decompensated and there seemed to be very little difference as I remember between those cirrhotics and normals

Best What does the pseudocholinesterase do Dr Bollman in hepatectomized animals?

Bollman In dogs surviving hepatectomy and remaining in good condition for twenty four hours we have found no appreciable alteration in the plasma pseudocholinesterase Esterase (tributy rinase) also shows no definite changes after hepatectomy nor is plasma amylase altered in this period Alkaline phosphatase may be increased after hepatectomy but many of our hepatectomized dogs show no change

Waterlow In a hepatectomized animal the period is too short to prove very much

Best Quite It would depend on how rapid the rate of dis appearance was

Bollman We have evidence of changes in the rate of protein formation brought about in the hepatectomized dog by changes in the amount of glucose made available to the animal without appreciable change in the cholinesterase of the plasma Figure 18 illustrates two experiments bearing on this point After liver re

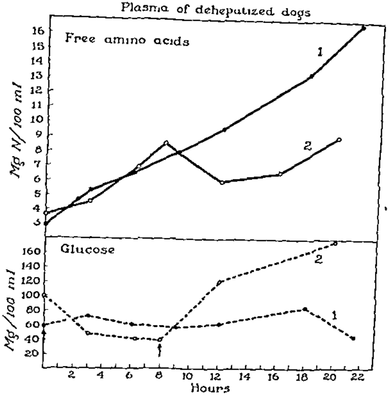


FIGURE 18

moval, at time zero, dog 1 received a continuous injection of glucose to maintain the blood sugar at approximately normal levels (0 14 grams of glucose per Kg per hour) *There was a progressive rise in* the free amino acid content of the blood Dog 2 received no glucose for eight hours, at which time hypoglycemic symptoms developed During this time, the amino acid content of the blood increased in a way similar to that of dog 1 At eight hours, dog 2 received 0 5 Gm glucose per Kg, at once followed by continuous injection of 0 24 Gm glucose per Kg The decrease in amino acids of the plasma is apparent With paper chromatography and some direct determinations of individual amino acids, we found that the changes in individual amino acids of the plasma were approximately in proportion to their concentration as found in normal plasma glutamine being the most abundant and showing the greatest changes The change in the free amino acids of the muscle are similar to those in the blood although the quantity is considerably greater When the amino acids decrease in the blood and

tissues they do not appear as anything else (no urea formation after hepatectomy) so that we believe that they go back again in protein but we are unable to measure directly to prove this point There are then very definite changes in protein formation without concomitant change in cholinesterase of the plasma

Watson That is in twenty four hours?

Bollman There was no appreciable damage in plasma cholinesterase during twenty four hours after hepatectomy

We have additional experiments which indicate that in the hepatectomized dog insulin is a large factor in protein formation We find a somewhat faster accumulation of free amino acids in the blood of depancreatized dehepatectomized dogs than in those only dehepatectomized Administration of large amounts of glucose does not influence this rise as it does in the liverless animal with pancreas remaining The administration of insulin to the depancreatized dehepatectomized animal minimizes the rise in plasma amino acid and if the insulin administration is delayed until high levels of amino acids are present in the plasma there is a rapid fall in the amino acid content of the blood We do not know whether this increased protein formation by insulin is independent of or directly associated with the insulin effect on glucose metabolism The liver less hypoglycemic dog does not survive insulin administration quite long enough to establish this point

This protein formation is all extra hepatic and we have no quantitative measure of its importance as compared to the protein formation by the liver

Best Do you remember the paper by Lotspeich (26) who studied the disappearance of amino acids from the blood under the action of insulin Those were intact animals

Bollman Yes His work gives more assurance that the disappearance of amino acids under the circumstances I have described is indicative of protein formation

Watson Did you have anything which would prove that the albumin synthesis was actually increased in these twenty four hours?

Bollman We have never been able to demonstrate plasma albumin synthesis in the liverless animal

Watson Whether albumin was synthesized at all?

Bollman Protein synthesis occurred in the tissues but we did not measure any protein concentration It was only the disappearance of the free amino acids that we measured

Gyorgy Why did you say cholinesterase had nothing to do here with protein synthesis?

Bollman The plasma cholinesterase was essentially unchanged over periods of time when we altered the rate of protein synthesis

Gyorgy There is cholinesterase in other tissues

Bollman Yes, there is cholinesterase in other tissues I referred only to plasma cholinesterase

Best In other words, the plasma cholinesterase would be a straight line right across, while the other would change

Bollman While the protein synthesis may vary

Gyorgy The protein synthesis doesn't happen in the plasma

Bollman I hope I did not infer that the protein synthesis occurred in the plasma Our only point was that protein synthesis could vary without comparable variation in plasma cholinesterase

Gyorgy But there is cholinesterase in the plasma

Bollman Yes, but it does not vary under these circumstances

Gyorgy If there was none, then I would go along with you

Bollman That is a different story Cholinesterase may play a role in protein synthesis, but under our conditions the quantity of cholinesterase in the plasma does not reflect the rate of protein synthesis

Madden Might it not be equally valid to interpret these changes, that is, the decreases in plasma amino acids after glucose administration, or glucose plus insulin in depancreatized dehepatectomized dogs, on the basis of a decreased liberation of amino acids from protein catabolism That is just as likely an explanation as an increased rate of utilization of plasma amino acids for protein synthesis

Bollman Except that if protein hydrolysis ceased, the concentration of amino acids in the blood would remain unchanged, as the amount excreted is small

Madden Whether plasma amino acids rise or fall or remain at a steady level is a result of the rates of the two processes liberation into the blood stream and disappearance from it When a decreased plasma level occurs, it appears necessary to show that decreased liberation could not account for the drop before concluding that increased re utilization accounts for it

Bollman Actually, we have little information as to the quantity of protein being hydrolyzed and resynthesized, and what I have been talking about is the accumulation of amino acids when hydrolysis exceeds synthesis or the disappearance of amino acids

when synthesis is greater It was on the basis of other data that I believed protein synthesis was increased

Shorr. Are these animals being continuously hydrated to keep up their blood volume?

Bollman Yes, there is no change in blood volume.

Shorr I was thinking of the speedy release of amino acids in shock

Bollman No, the blood volume is essentially the same all the way through

Best Dr Waterlow, have you further remarks?

Waterlow I should like to make the point that quite obvious microscopic differences can be shown and yet there is very much the same enzyme pattern I have pictures which include a normal liver, one that is quite heavily infiltrated with fat, another that illustrates fibrosis, and one that reveals regeneration after centrilobular degeneration, and if I were shown the enzyme figures, I could not allot them each a section or tell from which liver they came The histology is much more sensitive than the biochemistry at the moment, much to my disappointment

Kniseley A fatty liver can have enzymes that work

Waterlow Yes, they do have enzymes Of course, the figures for fatty liver are expressed on a fat free basis

Popper In animals, we find chemical methods, especially enzyme determinations in the liver tissue, more sensitive for the recognition of liver cell damage than routine histologic methods As a pathologist I am sorry to state that

Best There is a great advantage in studies on animals Dr Waterlow was working under most difficult circumstances, with human cases, in whom a lot of the measurements that are relatively easy in animals cannot be done

Popper Yes, that is true There are two points, however, that I should like to stress First, a few enzymes have been determined in liver tissue, namely, esterase, alkaline phosphatase, and dehydrogenases the latter either with tetrazolium alone or with added substrates In principle, in liver cell damage, hepatic esterase and dehydrogenases go down and the alkaline phosphatase rises This finding has been, under various circumstances, more sensitive than the histologic picture Secondly, I would like to refer to the relation between blood and liver enzymes, especially esterase, based on recent studies of Koch-Weser and Huerga (27) In acute intoxication with bromobenzene, as well as carbon tetrachloride, the liver esterase distinctly drops, however, in the early stage of the intoxica-

tion, the serum esterase rises and drops subsequently We feel that the damaged liver releases esterase, producing the serum rise, and only after a few days does the serum esterase drop to subnormal levels, following a biphasic curve The relation between serum enzymes and liver enzymes is, therefore, more complicated than would appear on the surface

Kark Nitrogen mustard therapy also causes a rise in serum cholinesterase We feel that it is probably due to damage and release of cholinesterase from tissue cells

Waterlow Didn't Brauer and Root (28) find the same thing in the dog?

Popper Whipple (29) described an increased blood lipase level in hepatic intoxications as early as 1913 There is another old publication (30) describing low hepatic and high serum esterase in acute carbon tetrachloride and phosphorus intoxication

Schwartz In his opening remarks, Dr Waterlow pointed out that it would be nice to do some isotopic studies to determine turnover rates of various compounds in the liver, but that this was not feasible because of the large amounts of activity that would be required During the past few months announcement has been made of a marked improvement in techniques at the Radiation Laboratory in California, in which they are able to determine one disintegration per milligram of barium carbonate per minute with a one per cent accuracy, and are able to detect 0 01 disintegration per minute per milligram of barium carbonate, which is much better than anyone has been able to do till now

I have a report by the Atomic Energy Commission which points out that this improvement now makes possible the study of C^{14} labeled isotopes in human subjects, and that it is possible to do some of these studies by injecting into a human patient as little as 0 001 millicurie of C^{14} containing compounds

Best I feel that studies of the enzyme patterns in liver and blood and muscle is one of the most important spheres of advance that is going on at the present time There is a very great future, clinically and physiologically in measuring the amount and activity of these enzymes preferably simultaneously in blood and several other tissues

Stetten There was a paper some eight or ten years ago from the group at Duke in which in a choline deficient, fatty liver it was demonstrated that choline oxidase was markedly diminished and that succinoxidase, if I recall correctly, was not diminished when computed per gram of defatted dry weight of liver And it was

subsequently shown (31,32) that this inhibition of choline oxidase could be induced in normal liver slices by the addition of emulsified fat

Best In other words, you are suggesting that not only the activity in the biopsy specimen be studied but that, accepting Dr. Pollister's suggestion, substrate and inhibitors should be added to see what happens

Stetten. It could be done if the material were available

Best Dr Waterlow, have you any further comments?

Waterlow No, thank you I am very grateful for the discussion

REFERENCES

1 WATERLOW, J C Liver choline esterase in malnourished infants *Lancet* 1, *908 (1950)*

2 ——— Observations on the activity of some enzymes in the human liver *West Ind Med J* 1, *41 (1951)*

3 HOLTER, H Technique of the Cartesian diver *Compt rend Lab Carlsberg Ser chim* 24, *399 (1943)*

4 LINDERSTRØM LANG, K and HOLTER, H *Die Methoden der Fermentforschung* Bamann, E, and Myrback, K, Editors Leipzig, Georg Thieme, 1940 (Vol III, p 1946)

5 WATERLOW, J C, and BORROW, A Experimental observations on the Cartesian diver technique *Compt rend Lab Carlsberg Ser chim* 27, *93 (1949)*

6 BORROW, A, and PENNEY, J R Further observations on biological reactions by the Cartesian diver technique, with special reference to mammalian tissues *Exper Cell Res* 2, *188 (1951)*

7 ADDIS, T, POO L J, and LEW, W Protein loss from the liver during a two day fast *J Biol Chem* 115, *117 (1936)*

8 KOSTERLITZ, H W *Effect of dietary protein on liver cytoplasm Nature (London)* 154, *207 (1944)*

9 MILLER, L L Changes in rat liver enzyme activity with acute inanition relation of loss of enzyme activity to liver protein loss *J Biol Chem* 172, *113 (1948)*

10 ROSENTHAL O *et al* Arginase adenosinepyrophosphatase, and rhodanese levels in the livers of rats *J Biol Chem* 185, *669 (1950)*

11 SCHULTZ J On the nature of labile protein I The cathepsin II activity of the liver and kidney of the fed and fasted rabbit *J Biol Chem* 178, *451 (1949)*

12 WILLIAMS J N JR and ELVEHJEM C A The relation of amino acid availability in dietary protein to liver enzyme activity *J Biol Chem* 181, *559 (1949)*

13 ——— The effects of tryptophan deficiency upon enzyme activity in the rat *J Biol Chem* 183, *539 (1950)*

14 BARGONI N Enzymatic activity and protein amounts in the liver *Experientia* 7, *101 (1951)*

15 AXELROD, A E., and ELVEHJEM, C. A Xanthine oxidase content of rat liver in riboflavin deficiency *J Biol Chem* 140, 725 (1941)

16 AXELROD, A E, SOBER, H A, and ELVEHJEM, C A D Amino acid oxidase content of rat tissues in riboflavin deficiency *J Biol Chem* 134, 749 (1940)

17 POLLISTER, A W, and RIS, H Nucleoprotein determinations in cytological preparations *Cold Spring Harbor Symp, Quant Biol* 12, 147 (1947)

18 SCHNEIDER, W C Phosphorus compounds in animal tissues extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid *J Biol Chem* 161, 293 (1945)

19 HOLMES, E G, and TROWELL, H C Formation of hepatic glycogen in normal Africans and in those suffering from malignant malnutrition *Lancet* 1, 495 (1948)

20 HARRISON, M F The relation between polyploidy and the amounts of desoxynucleic acid per nucleus in the liver and kidney of adult rats *Nature (London)* 168, 248 (1951)

21 KOCH WESER, D, FARBER, E and POPPER, H Fatty liver with and without necrosis, histological and biochemical investigations *Arch Path* 51, 498 (1951)

22 MCCANCE R A WIDDOWSON, E M, and HUTCHINSON, A O The effect of under nutrition and alterations in diet on the choline esterase activity of serum *Nature (London)* 161, 56 (1948)

23 HILL K R Liver disease in Jamaican children *Liver Injury* Hoffbauer, F W, Editor Trans Tenth Conf New York, Josiah Macy Jr Foundation, 1951 (p 263)

24 JOHNSTON, R B MYCEK, M J, and FRUTON, J S Catalysis of transamidation reactions by proteolytic enzymes *J Biol Chem* 185, 629 (1950)

25 KELLEY, V C *et al* Labeled methionine as an indicator of protein in children with lipoid nephrosis *Proc Soc Exper Biol & Med* 75, 153 (1950)

26 LOTSPEICH, W D The role of insulin in the metabolism of amino acids *J Biol Chem* 179, 175 (1949)

27 POPPER, H, KOCH WESER, D, and DE LA HUERGA J Serum and hepatic enzymes in experimental liver damage *J Mt Sinai Hosp* 19, 256 (1952)

28 BRAUER R W and ROOT M A The relation between the esterase activity of the blood plasma and of the liver of the dog *Am J Physiol* 149, 611 (1947)

29 WHIPPLE, G H A test for hepatic injury blood lipase *Bull Johns Hopkins Hosp* 24, 357 (1913)

30 JOBLING J W EGGSTEIN, A A and PETERSEN, W The relation of serum esterase to liver destruction *J Exper Med* 22, 707 (1915)

31 BERNHEIM F The action of fatty acids on the choline oxidase of rat liver *J Biol Chem* 133, 291 (1940)

32 HANDLER P and BERNHEIM, F The choline oxidase activity of fatty livers *J Biol Chem* 144, 401 (1942)

MORPHOLOGY OF THE LIVER

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Department of Anatomy
The Chicago Medical School

AT THE BEGINNING, I want to say that I am sorry we are talking about the liver and not about some other organ, such as the lung, for example, because there is very little we can learn concerning the nature of man from the study of so perfect an organ as the liver If we studied the lung and compared it with the much more efficient and better organized respiratory systems of insects or of birds, we would realize that man is, in many respects, a rather miserable creature The liver, however, is one of those organs, one of those few organs, which have achieved perfection

I shall try to give a brief summary of my work on the liver. I have studied the liver from several aspects, namely from the stand point of its cellular structure, from the standpoint of its evolution, and from the standpoint of its embryology Dr Popper and I are now applying the knowledge gained in these fields to the study of the very interesting liver disease, cirrhosis However, I am only going to discuss microanatomy, evolution, and embryology at this time

Table XIII shows the range of the studies * I present it because if I tell you something about the liver of lower vertebrate animals, I am afraid you will say I am generalizing, that something seen in Necturus does not necessarily hold true for all the vertebrates, and Table XIII is my justification for being dogmatic if I have not time to prove every statement

The older authors described the liver of the lower vertebrates as a net-like tubular gland (Figure 19) By the application of solid-geometrical and statistical criteria and other criteria of three-dimensional analysis, it has been found that the liver of the lower vertebrates is exactly the opposite of this It is a continuous mass of cells tunneled by cylindrical lacunae which all together form the hepatic labyrinth which pervades the entire liver (Figure 20)

* The studies have been carried out by Elias and Bengelsdorf (1,2) and by Hickey and Elias (to be published)

15 AXELROD, A E, and ELVEHJEM, C A Xanthine oxidase content of rat liver in riboflavin deficiency *J Biol Chem* 140, 725 (1941)

16 AXELROD, A E, SOBER, H A, and ELVEHJEM, C A D Amino acid oxidase content of rat tissues in riboflavin deficiency *J Biol Chem* 134, 749 (1940)

17 POLLISTER, A W, and RIS, H Nucleoprotein determinations in cytological preparations *Cold Spring Harbor Symp, Quant Biol* 12, 147 (1947)

18 SCHNEIDER, W C Phosphorus compounds in animal tissues, extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid *J Biol Chem* 161, 293 (1945)

19 HOLMES E G, and TROWELL, H C Formation of hepatic glycogen in normal Africans and in those suffering from malignant malnutrition *Lancet* 1, 395 (1948)

20 HARRISON M F The relation between polyploidy and the amounts of desoxynucleic acid per nucleus in the liver and kidney of adult rats *Nature (London)* 168, 248 (1951)

21 KOCH WESER, D, FARBER, E, and POPPER, H Fatty liver with and without necrosis histological and biochemical investigations *Arch Path* 51, 498 (1951)

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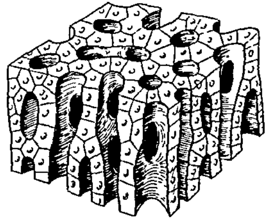


FIGURE 21 Stereogram of the liver parenchyma of mammals and Passeriformes (higher birds) Reprinted by permission from Elias H A re-examination of the structure of the mammalian liver the hepatic lobule and its relation to the vascular and biliary systems *Am J Anat* 85, 379 (1949)

The one cell thick liver plate consists of cells of various shapes Figure 22 shows four basic types octahedron (I), pentahedron (II), decahedron (III), and dodecahedron (IV) These types are not found frequently in reality, but each individual liver cell found in an actual mammalian liver presents features from two or three of these basic types The volumes of these types are very different from one another For example, a cell of type IV has almost four times the volume of a cell of type II Table XIV gives volumes and surface of the four basic types

The reason for the variation as I indicated previously, is that the shape, the vol me, and consequently the number of chromosomes [illegible]

[illegible] ear a hole located in [illegible]

geometrical and mechanical stability as exists in the mammals In the human liver however, the geometry of the cells is maintained under all kinds of conditions as shown in Figure 23 In the center is a piece of liver tissue where the blood pressure in the sinusoids

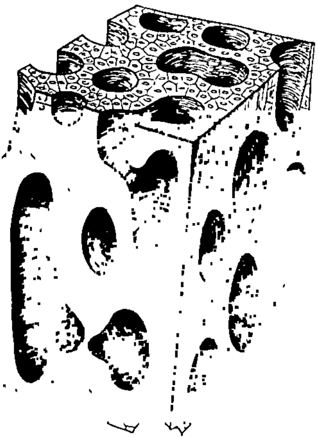


FIGURE 20 Stereogram showing the true structure of the lower vertebrate liver Two-cell thick walls separate lacunae Reprinted, by permission, from Elias, H, and Bengelsdorf, H The structure of the liver of vertebrates *Acta anat* 14, 297 (1952)

In contrast to this, the mammalian liver (Figure 21) consists of plates which are only one cell thick, and this must be a more efficient liver from the physiologic viewpoint, because each cell is in contact with the blood stream on at least two of its sides In fact, one-fifth, approximately, of the total surface of the cell in a mammalian liver is in contact with the blood stream while in the lower vertebrates, it is only one-eleventh

Among birds, two thicknesses of liver plates are found From this, we can conclude that birds possessing one-cell thick liver plates (Passeriformes = song birds) have acquired this characteristic independently of mammals We are dealing with a case of convergent evolution

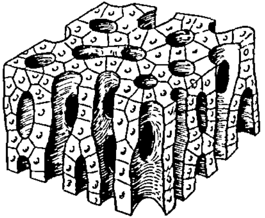


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The reason for the variation, as I indicated previously, is that the shape, the volume, and consequently the number of chromosomes of each liver cell are dependent on its location in the wall. Figure 22 shows the position of various cell types in the wall of liver plates

geometrical and mechanical stability as exists in the mammals In the human liver, however, the geometry of the cells is maintained under all kinds of conditions, as shown in Figure 23 In the center is a piece of liver tissue where the blood pressure in the sinusoids

Now we come to something which will please most of you since you are medical men If we zoologists study the lower vertebrates we do it only for the fun of it, because we are curious However, it so happens that accidentally or incidentally we find out things which are applicable to medicine Figure 24 is of a fatty liver, as

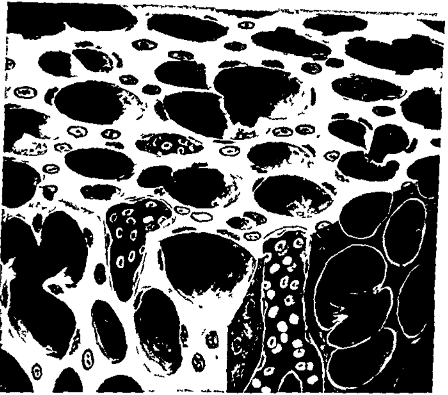


FIGURE 24 Stereogram of fatty liver showing lipodiastemata and the pseudosyncytium between them Reprinted by permission from Elias H Stereograms on *Cirrhosis and Fatty Liver* Decatur Ill Flint Eaton & Co 1951

described by Dr Hartroft (14) The large cavities are his lipodiastemata Between the lipodiastemata is a tissue which consists of sickly looking liver cells with indistinct or invisible cell boundaries which I want tentatively to call pseudosyncytium During the state of recovery (Figure 25) that is when the fat deposition decreases the lipodiastemata shrink Between them is seen the pseudosyncytium especially in the upper left hand corner This pseudosyncytium is reconstituting itself into two cell thick plates as is seen at the right

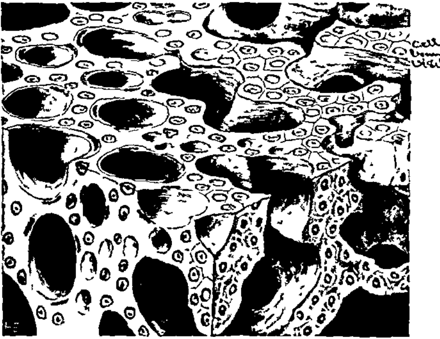


Figure 25 Reconstitution of liver tissue after fatty degeneration The pseudosyncytium (upper left) changes into two-cell thick plates (right) Reprinted by permission from Elias, H *Stereograms on Cirrhosis and Fatty Liver* Decatur Ill Flint Eaton & Co 1951

In other words during the regenerative phase, after fatty and other kinds of degeneration it frequently occurs that the human

about the pseudosyncytium if I understood you is parallel to some of the work that MacNider (15) did many years ago He found after an injury with carbon tetrachloride that was severe and almost fatal the liver regenerated with a plate of flattened syncytium very much like the embryonic type and was then resistant to injury

Popper The cells to which Dr Elias is referring are not as flat and narrow as the regenerative cells of Dr MacNider

Fremont Smith Somewhat of the same principle?

Popper The principle may be similar but the appearance is different

Elias I shall now come to a peculiar point concerning the evolution of the liver This concerns general principles of evolution which may be a surprise to many of you but are quite familiar to students of evolution In dogs, seals, water pigs, and carp that is, a few isolated species, are muscles which surround and project into the lumen of the hepatic veins and are arranged in a spiral or ring shaped fashion The existence of these muscles in the dog had been suspected in 1915 by Mautner and Pick (16), and were first actually demonstrated by our friend Dr Popper (17)

I recently had the luck to find the identical mechanism in the carp (18) If these muscles contract, stasis of blood occurs This contraction may occur along the entire hepatic venous tree These muscles contract completely during anaphylactic shock They also contract under chloroform and Nembutal anesthesia, not completely, but slightly They probably do not have any physiologic significance They are not necessary for life That this is true is proven by the fact that very closely related species, for example, the goldfish which is the closest relative of the carp, or the cat which is closely related to the dog and the guinea pig a very close relative of the water pig, do not possess these sphincters The useful functions which they may, at times, perform can well be performed by the outlet sphincters, which Dr Knisely (6) has described

These muscles can be absolutely insignificant or they can be ... are adaptive Others are

...

own bronchial muscles In the wilderness, certainly no person with asthma can survive until he reaches reproductive age (19)

Table XV brings us into the field of embryology, and here again I want to show that I have not been satisfied with studying the embryology of the human liver alone This is a list of the animals which I have already studied, or intend to study

... ... iman, in other words a pri ... (Figure 26) In the center is the gut (G) and ... diverticulum (D) From it arises a large plate of endodermal cells The omphalomesenteric vein is at O A little above it and also on the right side are capillary networks deriving from the omphalomesenteric vein The endodermal cells which emigrate from the gut wall wander into the spaces

TABLE XV

VERTEBRATES, THE LIVER EMBRYOLOGY OF WHICH

A HAS BEEN STUDIED

Class	Order	Species
AVES	GALLIFORMES	CHICKEN
MAMMALIA	MARSUPIALIA	OPOSSUM
	INSECTIVORA	HEMICENCETES
		SETIFER
	ARTIODACTYLA	PIG
	PRIMATES	MICROCEBUS
		MAN

B. WILL BE STUDIED IN THE NEAR FUTURE

Class	Species
TELEOSTEI	WHITE FISH
REPTILIA	TURTLE
AMPHIBIA	FROG
SELACHI	DOG FISH
CYCLOSTOMATA	LAMPREY

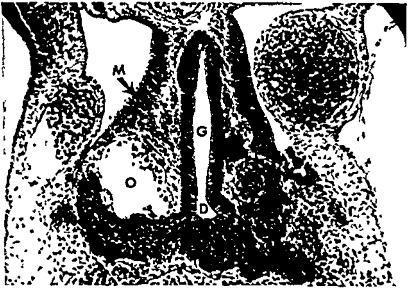


FIGURE 28 Liver anlage of Microcebus (prosimian) G gut D liver diverticulum M mesothelium O omphalomesenteric vein

between these vessels But that is not all The mesothelium (M) may also be seen that is, the mesodermal part of the embryo which is proliferating cells from it invade the septum transversum, the

space between the capillaries The liver of this animal is formed by the cooperation of endoderm and mesoderm

Figure 27 *shows the same thing in man It is a human embryo* At the bottom, the liver diverticulum is endodermal The entire territory of the septum transversum which contains small blood vessels, the future liver sinusoids, is invaded by endodermal cells but another part of the same network is invaded by cells which arise from the mesothelium by its proliferation (upper right) There is no boundary toward the substratum, the mesenchyme This mesothelium is actively proliferating and cells are invading the future territory of the liver These findings must make those among us who are interested in cancer a little suspicious that we have to look for mesodermal origin of some liver carcinomata

I shall now come to the story of the inversion of the theory of mammalian liver structure It was in 1947 that I made a movie relating to schistosomiasis Since the blood flukes spend a great part of their lives in the human liver, I decided to produce a film

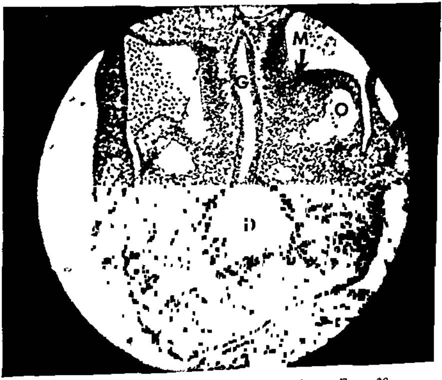


FIGURE 27 Liver anlage in human embryo Labels as in Figure 26

strip on liver structure as an introduction to the subject This seemed to be necessary because my students always had difficulty under standing the structure of the liver This film strip consisted of stereograms They were drawn according to the textbook descrip tions with no reference to slides The liver allegedly consisted of cords two cells thick surrounded by reticulo endothelium and bathed in blood I took the film strip to a meeting of anatomists in Montreal There eminent liver specialists saw it and said it was correct I then showed it to the members of the Anatomy Depart ment of the University of Chicago for the purpose of consultation There I received the first criticisms Dr William Bloom told me I should include a few photomicrographs to establish a transition between the imaginary picture and what the students would see Dr Knisely said that he had observed that the sinusoids are cylin drical Dr Bloom s advice was easy to take but I would not believe at all what Dr Knisely had told me The sinusoids cylindrical? I would show him that this might be true in frogs But in mam mals? Impossible! Complying with Dr Bloom s suggestion I found that Dr Knisely was right after all

At first I attempted to find cross sections of liver cords They should be numerous Oval oblique sections should be even more numerous

In order to get a longitudinal two cell thick section of a cord that contains a section only one cell wide you would have to be extremely lucky The cords would have to be very straight and you are only allowed to touch one row of cells and not cut over into the other row But this is just what we find almost exclu sively Actual liver sections as you know from your own experience show long rows of cells recurring into themselves unending one cell wide They are sections of plates or walls connected with other walls and perforated to permit the passage of sinusoids (Figure 21)

Figure 28 shows the difference between a plate or wall and a cord At D is a plate and at A there are cords All real liver plates are perforated (C) And the diagram (B) shows what the textbooks said Could there be transitions between a network of cords (B) and a cribriform plate (C)? Yes Figure 28 C shows the basic shape of a liver plate but in congestion both in humans and in lower vertebrates a change may occur When there is congestion the holes in the plates enlarge because the blood vessels pass through them and when the holes enlarge part of the walls between them becomes stretched then the perforated plate changes into a net

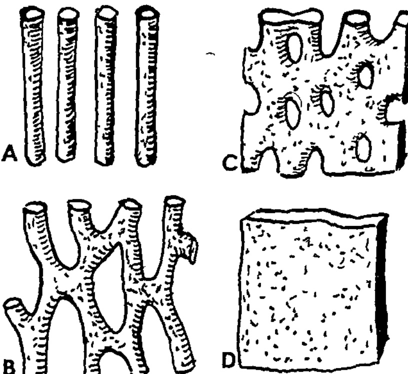


FIGURE 28 A cord B network of cord C cribriform plate D plate

work of cords I am sure that if you look through your own slides of congested livers you will find cross sections of cords in a few specimens

All these plates form a continuous wallwork as Figure 21 shows The walls (laminae hepatis) separate the lacunae from one another The lacunae hepatis are all connected with each other to form a vast labyrinth the labyrinth is hepatis (Figure 21) This liver labyrinth pervades without interruption the entire liver In it is suspended the equally vast network of sinusoids as Figure 29 (upper half) shows It is important to keep in mind that the lacunae (portions of the labyrinth) are spaces which contain the sinusoids but they are not identical with the sinusoids A chondriocyte may or may not fill its lacuna in the cartilage matrix In similar fashion the sinusoid may at times fill the hepatic lacuna and at other times be surrounded by the Disse space The hepatic labyrinths and the network of sinusoids are continuous throughout the liver and each exists only in the singular So really only one vast and multiple

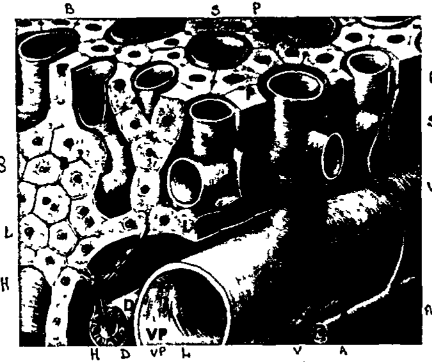


FIGURE 29 Stereogram showing the relationship of the liver parenchyma to the biliary and vascular systems A hepatic artery B bile canaliculi D bile duct H canal of Hering L limiting plate P intralobular parenchyma S sinusoid V inlet venule VP portal vein

branched liver plate exists a gigantic branched sheet of simple cuboidal epithelium As a labyrinth and a network of sinusoids, this one liver plate pervades the entire liver

This study has led to a complete reversal of the concept of liver structure While we formerly believed in cylinders of liver cells surrounded by blood we see now that the liver consists of cylinders of blood surrounded by liver cells

The portal canals (lower part of Figure 29 and center of Figure 30) are tunnels in the continuous mass of liver tissue They are surrounded by the limiting plate The limiting plate (lamina limitans) consists of hepatic cells which often are a little thinner and stain heavier than the intraparenchymal liver cells

Dr Grafflin tells me he has found a limiting plate beneath the capsule I was not astonished to hear it Near the hilus as Figure 31 (right) shows blood vessels and ducts run in a large mass of con



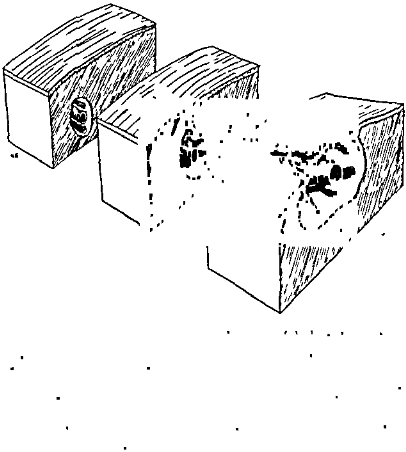
venule VP portal vein

Section along the ...

nective tissue This whole mass of connective tissue which contains the vessels and ducts sinks gradually into the liver (Figure 31 [center]) and when it is entirely surrounded by liver tissue it has become a portal canal (Figure 31 [left]) This portal canal as you remember from Figure 30 is surrounded by the limiting plate

It is quite obvious from Figure 31 that that limiting plate must be continuous with the surface of the liver just as the periportal connective tissue is continuous with the capsule so that there is but one limiting plate in an entire liver This limiting plate also extends to the other side surrounding the hepatic veins but they are not

Not much more was known about the distribution of the blood vessels than about the structure of the parenchyma when that film strip was made in 1947 So in order to produce a reliable piece for visual education the vascularization of the liver had to be worked



out almost from scratch (Figure 32 is the pattern of the portal vessels the portal vein runs in a clear space, the portal canal, short inlet venules arise from it These have sphincters, as Dr Knisely (6) has shown In this picture, there is a place where I assume the sphincter has just been closed, and another place where such an inlet sphincter is to be closed Due to the play of these sphincters, the blood flow is in continuous change This information we owe to Dr Knisely)

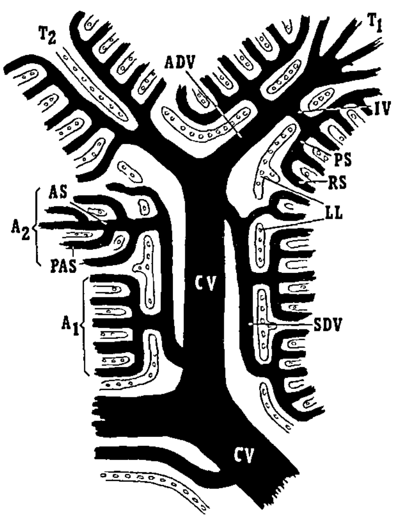
(The bile ducts are surrounded by a plexus of capillaries connected with the hepatic artery and with the portal vein It has been said that this plexus receives blood both from the portal vein and



FIGURE 32 Liver of rabbit India ink injected into mesenteric vein during life A distributing portal vein from which many inlet venules arise is seen in longitudinal section Inlet venules and the sinusoidal network are seen Sinusoids convey blood toward central vein

the hepatic artery Figure 32 proves that it does not receive blood from the portal vein because this specimen from a rabbit, was injected during life through a mesenteric vein If India ink is injected into a tributary of the portal vein in a living animal, the periductal plexus never shows However, the plexus will show if the mass in a dead liver is injected Then it can flow backward

Figure 33 is a diagram which shows the mode of branching of the portal vein I have the impression that the largest veins shown, the conducting veins (CV), are not located within the liver but in the indentations of the capsule as seen in Figure 31 at the right The small distributing veins (SDV) vascularize the neighborhood of the capsular indentations Those portal veins which are inside the liver (i e, in a cylindrical portal canal) are probably all of the axial distributing type (ADV) Recent observations of corrosion specimens of human and dog liver show that the blood flow in many of the small distributing veins (SDV) is directed opposite to the flow in the conducting veins (CV)


T2
T1
ADV
IV
PS
RS
AS
LL
A2
PAS
CV
A1
SDV
CV

[illegible]

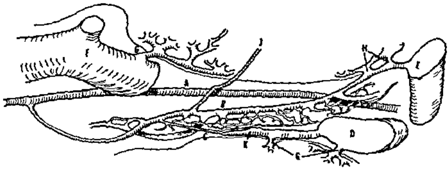


FIGURE 34 Arterial and portal vessels in portal canal of cat injected

(We come now to the arterial distribution Figure 34 shows a portal canal from the liver of a cat injected with different colored materials in the portal vein and hepatic artery The artery (A) can be seen It sends off an arterial capillary (B) This has no muscles around it but probably has contractile endothelium From the *arterial capillary is given off the periductal plexus This plexus by* the way is doubled around larger ducts Through this periductal plexus the *artery communicates with the portal vein These con*nections are the internal roots of the portal vein Branches go deep into the lobules from the artery (Figure 34 J))

I have a friend in the Midwest who does not want to believe that there are intralobular arterioles and arterial capillaries I do not know why *for I do not think it is such a terrible thing that* intralobular arterial capillaries exist He told me a few weeks ago *I look at thousands of livers every month and I have never seen* such a thing I said to him *If you looked at only one per year* and did it thoroughly you would certainly see them

I told this story to Dr Popper and he said Lets make a test" He pulled out the first normal liver slide he could get and we went to his photomicrographic equipment Within seven minutes Dr Popper located ten and photographed five intralobular arterial capillaries and *the time between taking one photograph and* locating the next one under high power ranged between five and fourteen seconds (It must be admitted however that very often these arterial capillaries are extremely small and hard to find They often are contracted in fixed livers and have a diameter of only 3 microns)

Figure 35 is a reconstruction Only the sinusoids are not reconstructed but drawn from injection preparations At the bottom is seen a portal canal *The portal vein* (P) ends at the right the artery (A) does not end but goes deep into the lobule and dis



FIGURE 35 Terminal blood vessels and ducts P portal vein A hepatic artery D duct S sinusoids Portal vein hepatic artery with the intralobular arteriole and arterial capillaries as well as the duct and the intralobular cholangioles are drawn from a reconstruction from 35 serial sections of a horse liver

tributes blood to all levels (Some of the arterial branches empty into the periportal sinusoidal network but more go into the interior There are sphincters at the narrow points in this picture which regulate the oxygenation and they are of clinical and physiologic importance For example the phase of lymph production can probably be influenced by contraction of arterial sphincters)

(Alongside each intralobular arteriole there always runs a little duct (D) The ducts never end blindly but form loops or are connected with far off ducts in other portal canals)

A few years ago I presented this reconstruction But I also presented other alleged evidence for the central arterial supply which was incorrect the Chrzonszczewsky (10) experiments which I had repeated were based on a fallacy So I had to repeat the whole study of the arterial end branches doing it differently This time

In the paper 'Selective Phagocytosis' by Knisely, Bloch, and Warner (6), we presented Mall's Figure exactly as he had drawn it originally except that we changed the stippling Mall's original Figure was stippled rather uniformly so that it did not present the details of that most important segment of the liver lobule in sharp contrast The only change we made in the Figure was to stipple each anatomical portion so that it stood in sharp contrast to each other part of the Figure The Figure shows that the perisinusoidal space is directly continuous (without interfering membrane) into the perilobular lymphatic* The perisinusoidal space and the lymphatic drainage of the liver lobule and changes in the width of the perisinusoidal space are described in 'Selective Phagocytosis' (6) The spaces are like the spaces between the leaves of a closed book A potential space is present When the sinusoid membranes are leaking fluid rapidly, this fluid passes out into the perisinusoidal spaces and forces the sinusoid lining away from the hepatic cells, whereupon the space is a real space and is no longer a potential space †

Elias The next few Figures will form the conclusion of this presentation (I want to show that liver lobules really do not exist as [illegible]

[illegible] tributary of a hepatic vein [illegible] nditions that is when the [illegible]

* See also Bolton and Barnard (23)

† Editor's Note Dr Elias added the following comments Referring back to the paper by Knisely Bloch and Warner (6) one finds the following passage "The lymph [illegible] it pre [illegible] the a [illegible] pass [illegible] must [illegible] liver [illegible]

Thus evidence of lymph production is presented but no evi[illegible] open commun[illegible] into the liver

Francis [illegible] ound that the consisting of [illegible] "peril bular dissolved dye [illegible] but that the [illegible] "All

very fine lymph vessels which accompany the [illegible] see Figures 38 and 39 The spaces of Mall and of Disse were entirely free from ink see Figures 38 and 39 of my paper of 1949 (21)

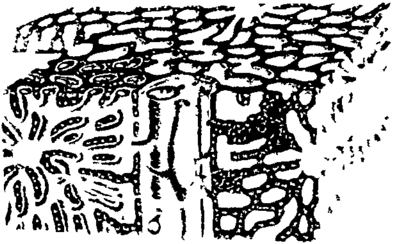
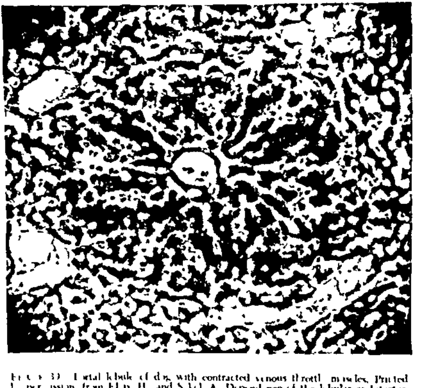


FIGURE 38 Synopsis of microanatomy of liver Reprinted by permission from Elias H Morphology of the human liver *The Liver Lobule* Skokie Ill, G D Searle & Co 1949

blood pressure is higher on the portal side than on the hepatic venous side. However, if the blood pressure in the hepatic veins is elevated or that in the portal veins decreased, the lobular pattern will become inverted. Lobules will then center around the portal canal, while the former central veins will be found at the periphery. Normally, the blood pressure in the portal veins is higher than in the central v[illegible]

straightens them [illegible]
ished i e if it [illegible]
lower on the p[illegible]
neighboring cen[illegible]
plenty of evidence for this)

This inversion of the lobular pattern occurs in the following cases



F[illegible] 33 [illegible]ortal lobule of dog with contracted venous throttle muscles. Printed [illegible] permission from [illegible] H and S[illegible] A Dependence of the lobular architecture [illegible] the parabiliary blood pressure gradient. *Anat Rec* (In press)

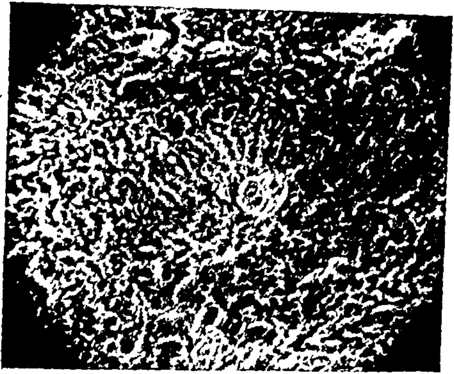


FIGURE 40 *Portal lobule of man with passive congestion Printed by permission from Elias H and Sokol A Dependence of the lobular architecture of the liver on the parahepatic blood pressure gradient Anat Rec (In press)*

I By elevation of hepatic venous pressure
  a) In dogs with contracted hepatic venous throttle muscles (Figure 39)
  b) In human passive congestion (Figures 40 and 41)
  c) In human active congestion due to central toxic necrosis
  d) When ligating a hepatic vein the reversal occurs in two minutes After release of the ligature the original hepatic lobules are restored in 30 seconds

II By lowering or total elimination of portal pressure
  a) In Zahn s infarct
  b) In certain obstructions of small branches of the portal vein such as shown recently by Hamperl (25)
  c) In obstructive jaundice i e when engorged bile ducts press on the portal veins thus reducing the portal pressure
  d) When ligating the portal vein and hepatic artery, the inversion takes place in about 3 minutes (Figure 42)

Figure 42 shows the liver of a cat Its portal vein and hepatic artery were ligated Then India ink was injected into a femoral

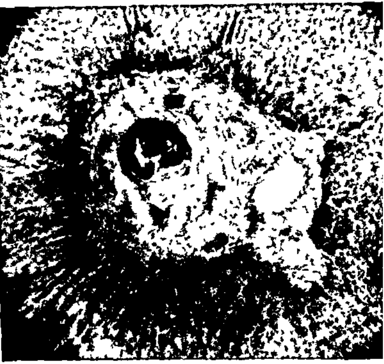


FIGURE 41 Portal canal from which liver plates radiate Same case as Figure
Printed by permission from Elias H and Sokol A Dependence of the lobular ar
tecture of the liver on the porta-hepatic blood pressure gradient *Anat Rec* (In pr

vein The ink passed retrograde into the liver through the hep
veins

Rappaport How long did this animal survive?

Elias We killed it as soon as we had the reaction

Rappaport There is always the possibility of getting this In
ink through the collateral veins

Elias Not in this region At other places yes Where those
lateral veins exist there is no inversion of the lobular pattern

(A rabbit was first observed normally under light anesthesia
ligated the hepatic vein and released the ligature Then we lig
the portal vein and hepatic artery Finally we killed the an
by an anesthetic With every change of ligature we got inver
of the lobular pattern These structural changes are reversible
the blood pressure gradient is changed in these vessels The char



FIGURE 42 Portal lobule of cat Portal vein and hepatic artery ligated India ink injected into femoral vein Ink appears through retrograde flow from vena cava, in the former central veins The white spot in the center is a portal canal. Printed, by permission from Elias H and Sokol A Dependence of the lobular architecture of the liver on the parahepatic blood pressure gradient *Anat Rec* (*In press*)

can be seen when observing or photographing the surface of the liver)

I discovered that the internal gross anatomy of the liver is not described in any textbook Therefore, Dr Petty and I undertook the task of mapping the blood vessels and ducts in the liver of human adults and fetuses (26) We hope that our findings will help surgery When we were almost at the end of our studies, I discovered by accident, in a pharmaceutical advertising magazine, a drawing made by Francis Glisson in 1654 showing in finest detail every little constant vessel in the human liver, identical with our



Figure 43 Stereogram of liver in Laennec's cirrhosis Reprinted by permission, from Elias H *Stereograms on Cirrhosis and Fatty Liver* Decatur, Ill, Flint, Eaton & Co 1951

findings His excellent work had been forgotten For almost 300 years, the anatomy books showed a big blank inside the liver.

Figure 43 shows that we, Dr Popper and I, are now working in the field of cirrhosis We are employing the same stereographic methods in it as were applied to the normal liver

(I have employed, to a large extent, geometrical methods and considerations (27) As illustrated in Figure 44, a cylinder, when cut, will as a rule give sections which are round or oval but practically always circumscribed (A) A fiber, when cut, will yield a dot or a short line (B) A plate, when cut, will yield, depending on the angle a very long band, narrow or wide, according to the angle of sectioning (C) If a line is found in the section it usually is the section of a membrane (D) This information can be applied to the study of connective tissue in cirrhosis)

(Figure 45 shows two types of connective tissue in a cirrhotic liver Dots at the right mark cross sections of fibers The name fibrosis" for connecting tissue of this kind is not wrong However,

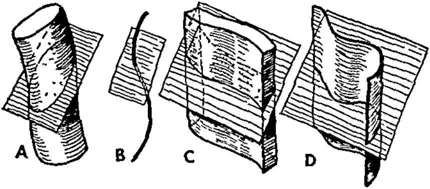


FIGURE 44

A An oblique section of a cylinder is an ellipse
B An oblique section of a fiber is a dot or comma
C An oblique section of a plate is a band
D An oblique section of a membrane is a line

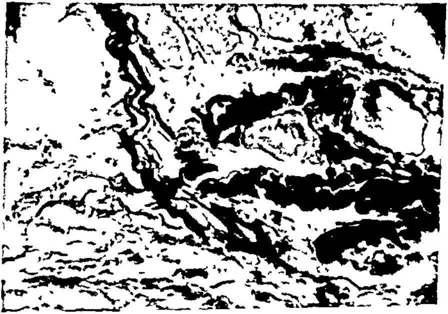


FIGURE 45 Connective tissue in cirrhosis Dots at the right are cross sections of fibers Lines at the left are sections of membranes

what is seen at the left are not fibers They are sections of membranes very long lines if they occur in great numbers in a section cannot be sections of fibers Sections of fibers look like what is at the right)

Tremont Smith Why do you say this could not be a fiber?

Elias Because if it were a fiber crooked as it is the chances of its running in this one plane and being cut along its entire length are so slim that such a possibility can almost be excluded If we find in a section most of the connective tissue in the form of these long lines it is a geometrical and statistical impossibility that they would be fibers

Knisely But there they are branching repeatedly, aren t they?

Elias They are not branching This membrane arises from apposition of several extremely thin membranes which we call "micromembranes"

Knisely A series of layers one right after the other?

Elias Yes

Knisely Have you traced this through the next histological section?

Elias Yes

Knisely How many series?

Elias This is from a series of 100 We always do 100 sections However since these membranes are corrugated their tracing through successive sections is often very difficult Thus in order to reconstruct these membranes we must use optical sections i e we focus each section low medium and high Figure 46 is reconstructed from 3 sections of 10 microns each focused at 3 levels This means it is reconstructed from 9 optical sections

This connective tissue is first laid down in the form of extremely thin micromembranes and later these aggregate to form larger membranes and more of these large thick membranes aggregate to form septa

As a conclusion I shall propose a new nomenclature for the proliferation of connective tissue The word "fibrosis" should be eliminated because we are not always dealing with fibers I would propose to use a noncommittal term like "syndesmosis" syndesma meaning connective tissue There are two kinds of syndesmosis membraneous from the Greek word membrane μεμβράνα* and rhammosis from the Greek word [illegible] meaning "a thread"

Best Dr Crafflin will you discuss this paper?

*μεμβράνα however is not classical Greek It derives from the Latin, and occurs in this form only in the New Testament



FIGURE 46 Reconstruction from 3 sections of 10μ each focused at 3 levels i e reconstructed from 9 optical sections showing collagenous membranes in cirrhosis arising from central toxic necrosis

Grafflin I think that all of us owe a deep debt of gratitude to Hans Elias Most of us when his work appeared on the laminar structure of the mammalian liver frankly wondered whether we had not been overpaid for a great number of years for teaching medical students who could not possibly have confirmed what we said about liver cell cords We were earnest about our teaching and we were earnest about the liver but this does not alter the fact that we did not sufficiently question the statements made in the textbooks Now I think we begin to comprehend the structure of the mammalian liver Dr Elias has driven home the fact that there is in mammals (including man) a laminar rather than a cord structure of the liver He has driven home the fact that these laminae or plates are typically one cell in thickness Certainly by the methods we are now using we can repeatedly confirm these arrangements

I know Dr Elias would agree that in some parts of this work it has been a great pleasure to him to have had the benefit of the advice of Frederic T Lewis Elias three dimensional analysis of the organization of the bile canalicular system which clearly shows Dr Lewis's influence has been highly contributory Again we begin to see things more clearly and can look beyond the fragmentary pictures of bile canaliculi as seen in ordinary sections to a three dimensional picture of the canalicular system which is largely continuous Frederic Lewis's work on the shapes of cells is beautiful work done by an extremely thoughtful individual whom some of us had the pleasure of knowing I am delighted to see it come forward at this time into an area where a wider group of physicians and biologists will become acquainted with it

Elias recent announcement about the participation of mesoderm in the development of the liver will come as a surprise to many anatomists who have been teaching students for many decades in terms of a strictly endodermal origin On the other hand if one thinks as most of us do of these various germ layers as transitory units of embryonic organization perhaps it is not philosophically as startling as it might otherwise be He now tells us that liver lobules dont exist and I suppose that the pig will scold him for that

Elias May I interrupt you for a moment Dr Grafflin? The pig in my mind presents a case of congenital nonprogressive cirrhosis

Grafflin For years in teaching medical students we have been dropping the pigs liver into acid until it looked and felt soft enough transferring it to water and then shaking out literally thousands of lobules for a class The students can study with ease the multifaceted polygons or lobules isolated in this way I am not sure the pig is right if he tries to scold you Hans but I think he is the one who is liable to raise his voice

The so-called perisinusoidal (Disse) spaces fascinate me I have looked at many living livers in frogs and mammals and I have never seen them But Dr Knisely is infinitely more acquainted with livers than I am and I would be most interested to hear his views I deduce from the literature that all thoughtful students of the liver are philosophically convinced of the necessity for these spaces in the normal living animal Although I have searched diligently I have never seen them But I too am philosophically convinced that the hepatic lymph which has to come from somewhere probably originates in large measure in these perisinusoidal spaces

It appears that Dr Elias was not surprised by our observations on the subcapsular laminae I confess that we were When we started our work upon the mammalian liver, we were faced with pictures which we could not comprehend until we satisfied our selves as to the existence of the subcapsular laminae We regard this as a trifling advance as far as the structure of the liver is concerned, but it was major in its importance to us

You tell us that the liver has achieved perfection I am not sure that nature would agree with you there You said you were sorry you were talking about the liver tonight I am not sorry at all, and I think most of us are not We have learned a very great deal from what you have said

Lagerstedt There is one point of view on the problem which I should like to stress In the first place, I am impressed by this demonstration of the nonexistence of liver lobules in the meaning that the word has been used before

I shall come right to the point with the following example, which I think most of you know very well The accumulation of glycogen in the liver can be followed histochemically In the rabbit at one time, the glycogen will be found accumulated around the central veins in all the lobules, at the same time, the bile acids can be shown with the method of Forsgren (28) to occupy the cells in the periphery of the lobules At other times during the twenty four hours, all the cells will be filled with glycogen and no cells with bile acids Thus, there are three main possibilities for localization in the periphrey or in the center of the lobules or within all cells seen After Dr Elias' presentation, the question arises Can observed changes in position of substances like those exemplified above be caused only by an inversion in the pattern of the liver?

Elias Not at all You are entirely right The only thing which is wrong is the way you describe the zones It would be better to distinguish the three zones, or the two extreme zones, as paraportal and parahepatic Both venous systems interdigitate, and there is a region which is closer to the portal tree, that is, on the proximal side of the blood stream There is another region on the distal side of the blood stream which is closer to the hepatic tree Therefore, your facts do not at all contradict this observation There is an essential difference physiologically, between the paraportal and the parahepatic zones The hepatic lobules exist, as I said, under normal blood pressure conditions, and usually we have normal blood pressure conditions The only [illegible] at it is [illegible] t a [illegible] er manent structure It is invert[illegible] e an [illegible] nve

Lagerstedt Yes that is the point I wish to stress

Knisely There are a number of subjects concerning which I should like to talk First concerning the evidence that the liver does not have does not consist of definable lobules certainly I would agree completely that in many specimens of liver prepared by several different techniques there is no visible or detectable evidence of a specific morphologic border acting as a boundary or delimiting each lobule This however does not mean that lobules do not exist Permit me to call your attention to page 531 of the first edition of the *Textbook of Histology* of Maximow and Bloom (29) This is the edition which is most useful as a reference book for it contains the largest amount of carefully collected and winnowed and carefully written detail Professor Maximow wrote

Lobule of the Mammalian Liver The mammalian liver shows grossly a more or less distinct mosaic of regular polygonal small areas This can be seen on its peritoneum-covered surface as well as on a sectioned surface Each of these areas represents a small architectural unit or lobule 0 7 to 2 mm in diameter The periphery of the lobules is translucent and gray while the center is brown In certain pathologic conditions connected with venous hyperemia the center of each lobule is dark red or brownish while the periphery is pale this produces a mottled appearance (nutmeg liver)

"In man the outlines of the lobules are usually not distinct because the connective tissue partitions between them are poorly developed In the pig polar bear and camel on the contrary each lobule is completely surrounded by a layer of connective tissue and the lobulation is accordingly very distinct (Figure 399) When a freshly sectioned surface of the liver of one of these animals is scraped with a knife the soft tissue is squeezed out of the lobules and the remaining partitions give the impression of a honeycombed structure In cirrhosis of the liver in man the connective tissue may be greatly increased and the usual lobulation completely distorted

In the salivary and pancreatic glands the lobulation is distinctly visible to the naked eye and depends on the distribution of the branches of the ducts In these glands each lobule represents a mass of glandular tissue drained by a duct of a certain order and size The liver lobules however do not depend on the duct system they depend on the distribution of blood vessels This is clearly seen in microscopic sections of a liver whose blood vessels have been injected with colored masses (Figure 406)"

The very beautiful pictures of histologic sections in the new *Atlas der normalen Histologie und mikroskopischen Anatomie des Menschen* (30) show the liver of the pig and that of the injected liver of the rabbit The pig liver shows the connective tissue septa clearly and the rabbit liver shows the delineation of the lobule border by means of vascular injection

In the case of the pig F P Johnson in 1918 (31) using Huber's technique to dissolve the connective tissue septa was able to isolate and show the shapes and sizes of the lobules of pig's livers

As noted above in a number of species including man there is as yet no definable morphologic border separating adjacent lobules However this does not mean that the direction of blood flow is not controlled within the lobule In our own laboratory we have examined under the microscope the living transilluminated livers of more than 5000 frogs and the living livers of a much smaller number of mice rats and Rhesus monkeys All of our observations agree that there is a precisely controlled direction of flow within each sinusoid or set of sinusoids and within every part of the lobule Under perfectly healthy normal conditions the directions of flow are from the periphery of the lobule toward the center following in general the direction of flow deduced by Mall (24)

There is a unidirectional flow in the sinusoids with a few minor variations from time to time Under normal conditions, with normal pressures, each and every sinusoid has a flow almost entirely from the periphery of the lobule toward the center

From our evidence I do not believe that as a part of healthy normal physiology a central vein becomes an afferent vessel or a portal vein becomes an efferent vessel. As a part of physiology a reversal of direction of flow through the lobules does not occur But we have observed as a part of known pathology for example when some vessels are plugged with masses of agglutinated blood cells a reversal of flow which can and does take place through major segments of lobules

the efferent vessels
ection of blood flow
surgically inverted

Smetana This is an important point because we have to deal first with normal structure

Knisely We must know all phases of the normal and all phases of the pathologic and the transitions between them and each phase must be labeled correctly

Elias Arey (32) reported years ago that in certain animals and also in humans there are occasionally portal lobules and he thought these were individual or regional variations They were not It depended on which vessel he cut first when taking out the liver I don't have to explain this You understand If you first cut the portal vein
lobule If
lobule and
the time of fixation

Knisely There was a long academic argument between the adherents of Mall (22,24 33) and of Kiernan (34) as to which type of lobule the one centered around the central vein or the one centered around the portal venule should be considered "the liver lobule"

Elias And Mall especially based his argument for the portal lobule on the findings by Brissaud and Sabourin (35) in the seal The seal happens to be one of the animals with hepatic throttle muscles and the specimens obviously were fixed while those muscles were contracted

Watson Perhaps it is stupid but I don't quite understand how the pattern is reversed by tying the veins because surely as soon as one vein is tied the pressure must equalize throughout the liver

Elias Yes this is true but normally there is a gradient portal pressure high hepatic pressure low

Watson Yes normally

get a uniform distribution very rapidly

Elias It takes minutes to get inversion and only thirty seconds after the ligature is taken off to get the reconstitution of the normal pattern

Deysach (36) injected India ink into the hepatic veins and his illustrations show this inversion clearly not that he paid any attention to it However if you are prepared to suspect it you will notice the inversion whenever it appears Deysach cut out the whole liver injected India ink through the vena cava and of course the injection pressure provided the inversion of the pattern

I should like to make one point concerning these throttle muscles which I forgot but which is important I believe Because of the existence of these throttle muscles the dog should be ruled out in

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Knisely There was a long academic argument between the adherents of Mall (22,24 33) and of Kiernan (34) as to which type of lobule the one centered around the central vein or the one centered around the portal venule should be considered "the liver lobule

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Elias Yes this is true but normally there is a gradient portal pressure high hepatic pressure low

Watson Yes normally

Elias If they are equal you have elevated the hepatic venous pressure relatively and relatively lowered the portal pressure It is not a matter of absolute pressure but of the gradient

Watson I dont quite see that I would have thought you would get a uniform distribution very rapidly

Elias It takes minutes to get inversion and only thirty seconds after the ligature is taken off to get the reconstitution of the normal pattern

Deysach (36) injected India ink into the hepatic veins and his illustrations show this inversion clearly not that he paid any attention to it However if you are prepared to suspect it you will notice the inversion whenever it appears Deysach cut out the whole liver injected India ink through the vena cava and of course the injection pressure provided the inversion of the pattern

I should like to make one point concerning these throttle muscles which I forgot but which is important I believe Because of the existence of these throttle muscles the dog should be ruled out in

very many cases as an experimental animal, because whenever blood pressure plays any role, the contraction of these muscles will store blood in the liver That is, the peripheral blood pressure will be lowered These muscles do not only react to histamine but to other agents also, as, for example, chloroform and Nembutal I don't mean to rule out the dog for studies of the liver, but for studies on any other organ, whenever the blood pressure plays a role, no dogs should be used because the hepatic throttle muscles distort the picture

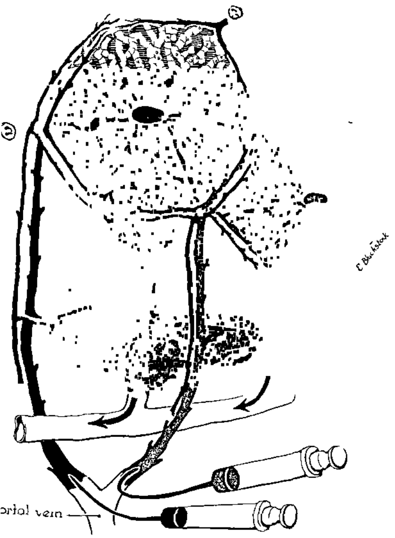
Best I shall ask Dr Rappaport for his comments

Rappaport I must say that I enjoyed very much Dr Elias' expert guidance through the liver, which he sometimes calls the "Labyrinthus Hepaticus" (21,37) However, to find our way through this labyrinth, I think we shall need two threads of Ariadne, a red one of circulation and a green one of bile secretion

Two years ago we became interested in the study of the liver structure mainly to further our understanding of the lesions we observed after experimental ischemia of the liver The reported "central" necrosis did not make any sense to us, as we know from clinical evidence and by experiment, ischemia has its most deleterious effects on the periphery of an organ As the small units of the liver represent in miniature the functions of the entire organ, we expected them to behave accordingly Because they did not behave so and showed lesions centrally instead of at the periphery, we questioned Kiernan's (34) concept of the lobule and started to investigate it We did this by using some injection methods Together with Drs Z J Borowy W M Lougheed and W N Lotto, we injected differently colored gelatin masses through two branches of the portal vein the hepatic artery or bile duct

Figure 47 illustrates the technique used, and at the same time, it gives a rough blueprint of the liver's architecture If we inject simultaneously two gelatin masses, a red one and a black one, of the same viscosity and under the same hydrostatic pressure, through two branches of the portal vein the liver will become colored and will show both colors, which will meet in an intermediate zone If, from this intermediate zone, a tissue block is cut, one small branch of the portal vein (B) brings in black and the other (R) brings in red color (Figure 48)

It may be expected that the color travelling along the portal channels will find its terminus in a microscopic area belonging to a terminal portal branch Because of the simultaneous inflow under the same pressure of the differently colored mass through a neigh



IGURE 47 Subdivision of the hexagonal lobule in liver acini The liver acini LA) are small berry like parenchymal masses situated around the trio of rminal branches of *portal vein hepatic artery* and *bile duct* branching out om a small portal space L A¹ = Horizontal section through a red colored ver acinus extending from one central vein to another

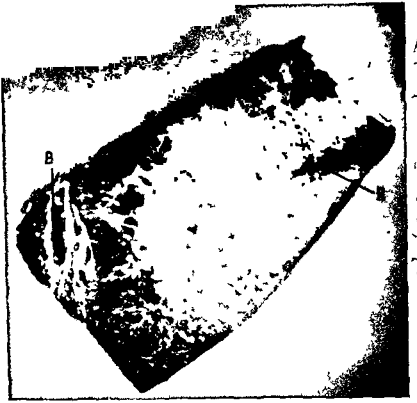


FIGURE 48 Tissue block from the intermediate zone B portal branch bringing in black gelatin mass R portal branch bringing in red gelatin mass

boring terminal portal branch the spread of the color by anastomotic sinusoids from one area to another is minimized

By using the same technique for injection of two branches of the hepatic artery or two branches of the bile duct we were able to define a liver unit which we call "liver acinus" The liver acinus is a small mass of parenchyma around the terminal branches of the hepatic artery, portal vein, and bile duct, branching out from small portal spaces It is designated as La in Figure 47 a section of the red mass that extends into two adjacent hexagonal fields The small berry like mass which is indicated by the letters L^1a^1 is supplied with blood by the two afferent vessels and drained of its secretions by the terminal bile duct which runs along with them

The acinus reaches two central veins, and in this way two afferent vessels are drained by two veins, while in the hexagonal lobule six afferent vessels are believed to be drained by one vein This is contrary to a basic principle in vascular anatomy, that the number of the venous channels is predominant The liver acinus is drained by at least two veins at its periphery

A regular hexagonal field is formed by six halves of liver acini, while the other halves belong to the adjacent hexagonal fields However, in reality, the acini are not divided in two by the hexagonal fields, just the contrary The hexagonal field is broken down by different liver acini



FIGURE 49 Liver acinus in the liver of a rabbit The area clear of India ink is centered around the trio of terminal branches of portal vein (PV), hepatic artery, and bile duct The latter is seen in this section cut longitudinally

Figure 49 is of a rabbit's liver injected with two colored masses through branches of the portal vein A branch in a portal space through which the red color is brought in can be seen The color extends from one central vein to the other, outlining the shape of a liver acinus

Figure 50 shows a hexagonal field which was broken down in different sectors by the injection of different colored masses through



FIGURE 50 Hexagonal field broken down into differently colored sectors which represent parts of liver acini injected through terminal branches of the hepatic artery

two branches of the hepatic artery One small branch of the hepatic artery is seen bringing in black gelatin, and the other one red gelatin The colors delimit sectors in the hexagonal field

Figure 51 is a similar picture obtained by simultaneous injection of two colored gelatin masses under the same hydrostatic pressure into two branches of the bile duct One branch conveys black color into red hexagonal fields The spread of the black color in an acinous area can be observed extending from one central vein to another

Injection of the bile duct is more difficult to carry out than injection through the vessels In spite of the anastomosis between bile ducts that Dr Elias enunciated there is not such a free flow of the injection mass as through the sinusoids into the hepatic veins However, the bile canaliculi and the acinus that extends from one central vein to the other can still be outlined, as in Figure 52

In summary following the natural course of the channels that bring in the material necessary for function and the course of the channel that carries away the product the bile, we were able to



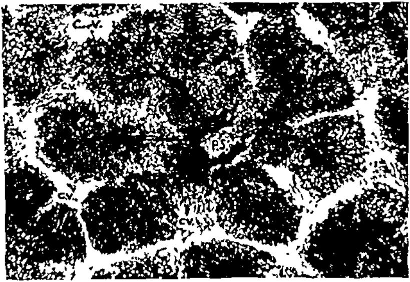
FIGURE 51 Dog's liver injected through the bile duct The spread of the black color extends into sectors of adjacent hexagonal fields Their central veins (CV) are surrounded by red colored areas (here of gray color)



FIGURE 52 The black areas injected through the terminal branches of the bile duct correspond to acini

outline an acinous unit in which structure and function are well correlated)

The study of the pathology of an organ helps in an understanding of its normal features)



with India ink The pathologic process small portal space C V central vein S, and Ridout J H Pathogenesis of Escape of lipid from fatty hepatic cysts into the biliary and vascular system *Am J Path* 27, 985 (1951)

Figure 53, from Dr Hartroft's collection on dietary cirrhosis, represents the liver of a rat injected with India ink into the heart It looks as if cirrhosis, with its heavy fibrous lines that have wiped out the sinusoids around the periphery of the liver acini, caricatures the normal features of these units Here, too, the hexagonal field

space, the axial vessels of the different liver acini may also be seen and at their periphery central veins, indicated as C V

Returning to the problem of ischemic necrosis, Figure 54 shows, at first glance that necrotic lesions are at the circulatory periphery of the liver acini The cells adjacent to the axial vessels indicated a a b b c-c, are the last to die and the first to regenerate The



FIGURE 54 Ischemic necrosis of a dog's liver after Eck fistula and ligation of the common hepatic artery. Rims of liver tissue have survived. They extend symmetrically (a a, b b, c c) around terminal vascular branches. X, necrotic area.



FIGURE 55 Necrosis in a dog's liver following experimental ischemia The necrosis is central due to the destruction of equally large peripheral parts of adjacent liver acini Note the regular rims of surviving parenchyma (R) around the axial vessels of the liver units

so called central necrosis is, in certain places, far from being central For instance, the area marked X

We know why these cells resist necrosis Olds and Stafford (38) have demonstrated that most of the arterial capillaries join the portal sinusoids in this area, close to the axial vessels

For Figures 55 and 56, I am obliged to Lieutenant Giges from the Army Medical Center in Washington, who repeated our experiments with ischemia of the liver for the production of hepatic coma I was amazed by the regularity of the islets of tissue that survived around the axial vessels of the liver acini In cases where several adjacent acini were affected equally by the cold bloom of

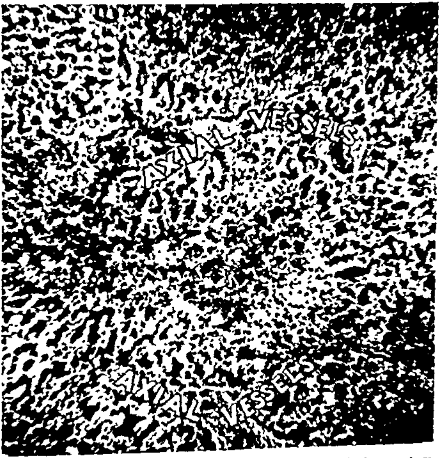


FIGURE 56 Ischemic necrosis in dog's liver The axial vessels of the liver acini are surrounded by equally wide cords of surviving cells They enclose bandlike areas of necrosis in which central veins can hardly be identified

anoxia and the periphery of the acini was destroyed and shriveled necrosis was regularly central However if one liver acinus was destroyed to a greater degree and the other to a lesser degree the central vein was not situated in the center of the necrotic area

In Figure 56 we could say that this band of necrosis is central but the barrier of living tissue that resisted the pathologic process is evident and from there regeneration will proceed

I think all our orientation should center around these axial structures which form the core of the liver acini This will permit a better functional approach to all processes that are going on in the normal and in the abnormal liver

Elias Dr Rappaport I believe Figure 56 shows evidence of regeneration because there are two cell thick plates

Rappaport How does our concept answer Dr Elias question of why we observe only unicellular and not bicellular liver cords between the sinusoids If we may reconsider Figure 47 for a moment we note that in whatever relation to the central veins of the Kiernan lobules sections may be made there will be always cut tangential slices of the grape like masses the axes of which have by no means a rigid geometric arrangement around the central veins These rounded parenchymal masses may overlap each other crisscross their axes they may even dislocate each other by differences in the individual growth processes The liver cords fuse of course and form plates which run between the sinusoids towards the terminal hepatic veins If we assume that each acinus is composed of many fused tubular cords we might compare each of these small units to a spherical brush. Each bristle would correspond to the canalicular lumen of a liver cord, and the cells of the cords would fill the spaces between the bristles. Six of these brushes surround the central vein in a perpendicular plane In whatever plane

ential sections of

l

ay of the bristles transversely and thereby display bile canaliculi delimited by two hepatic cells The chances are greater for hitting the liver cords tangentially and finding shorter or longer unicellular chains interrupted by adjacent sinusoids This conception is in agreement with the embryological development of the human liver from cords

We should not expect an ideal regular shape of the liver acini nor are they completely separated from neighboring tissue belonging to other terminal vascular and biliary branches Interdependence is a basic principle in our organism

Best Will you carry on Dr Popper?

Popper As a pathologist I am extremely grateful that I have had the chance for the last two or three years to work with Dr Elias and learn from him

There are several points to which I would like to give emphasis The first deals with Dr Elias concept of one cell thick liver cell plates instead of cords two cells thick This concept helped me to understand more clearly regeneration of the liver Based on the old concept of cell cords I always thought that in cases of necrosis of the liver cells the cords would grow in longitudinal or axial direction to fill out in single or double file the spaces in the preserved but empty fiber framework I came to learn that the liver cells may regenerate in transverse direction perpendicular to the axis of the plates, which results in several cell thick plates. Dr Elias calls that a blastema This is important because we understand much better now that such growing blastemas may, in cirrhosis, compress the hepatic veins and thus produce portal hypertension This fact briefly referred to in older papers by Kretz (39) and McIndoe (40) has been very well established recently by Kelty Baggenstoss and Butt (41) To me the principle of regeneration in perpendicular direction appears the most important pathologic application to date of the presented concept of liver cell plates I would like to use this opportunity to express my great happiness that a number of the members of this Conference are supporting the concept of Dr Elias To me personally it is a very great relief to feel on safe ground

My second point deals with the mesodermal origin of the liver I must confess that the first time Dr. Elias talked to me about this concept, I threw my hands up and told him that he was confusing me on every fact concerning the structure of the liver I had accepted until then Recently we have come to realize that mesenchymal elements may possibly play a role in hepatic carcinomas Such elements are especially common in the hepato-blastomas the congenital tumors of the liver We may have here again embryologic findings supporting pathologic and oncologic observations

My third point deals with the question of intralobular cholangioles a concept which has recently become very close to my heart It may be justified to spend one or two minutes on nomenclature for better understanding We all agree that the small canaliculi between the epithelial liver cells should be called bile capillaries and the ducts in the portal triads bile ducts Between the bile capillaries and the bile ducts is an intermediary portion which is

designated in the literature with different names such as canal of Hering ampulla junctional bile duct (the name applied by Dr MacMahon) or cholangiole

Drs Watson and Hoffbauer (42) have taught us the very important concept of cholangiolitic hepatitis assuming an injury localized in this particular area and resulting in regurgitation of bile through the damaged cholangioles For the past years many of us I amongst them have spent considerable effort in looking at liver specimens to find a specific lesion in or around the portal triads which would explain this cholangiolitic hepatitis Suddenly I learned from Dr Elias that these cholangioles may be not only around the portal triad but may form a wide network within the lobular parenchyma (Dr Elias has just handed me a note stating that Ebert in 1867 described these intralobular cholangioles Anyway I learned it from him) We therefore became interested in the possibility that these intralobular cholangioles may be the site of the injury in cholangiolitic hepatitis

A recent experimental observation was the second cause for our interest in cholangiolitis namely the pathologic findings in the livers of rats that result from prolonged dietary administration of ethionine

I should like to stress that my own interest in lesions produced by ethionine was markedly enhanced by something I learned at the Macy Liver Injury Conference two years ago Dr Davies (43) described pancreatic changes resulting from malignant malnutrition This stimulated us to look at the pancreas of ethionine intoxicated rats Dr Farber (44) found that ethionine an antagonist of methionine which presumably interferes with protein synthesis produces pancreatitis in rats Recently we observed following prolonged ethionine administration chronic pancreatic atrophy

(We (45) had known from acute experiments that ethionine produces fatty liver in female rats Dr Koch Weser (46) recently placed rats on a diet that contained about 0 4 per cent methionine in its protein component and added to this 0 5 per cent ethionine Rats on such a diet developed severe hepatic changes within four to five weeks These were characterized by diffuse degeneration and regeneration of the hepatic cells associated with a severe interstitial inflammation composed mainly of reticulo endothelial cells (Figure 57A) These changes justify the designation hepatitis With higher power lumina are made out between the liver cells and the interstitial inflammatory cells (Figure 57B) which we considered



FIGURE 57 Hematoxylin eosin stains Liver of rat which had been on an 0.5% *ethionine diet for 34 days followed by 10 days on a stock diet and subsequently for* 30 days on the 0.5% ethionine diet

A *Irregular arrangement of the liver cell plates without distortion of the lobular* pattern Individual liver cells and small groups of cells are separated by an interstitial infiltration composed of reticulo-endothelial cells surrounding cholangioles. (x90)

B The liver cells reveal signs of degeneration and regeneration resulting in great polymorphism The lumina lined by cuboidal epithelium are cholangioles (x285)

marked by x

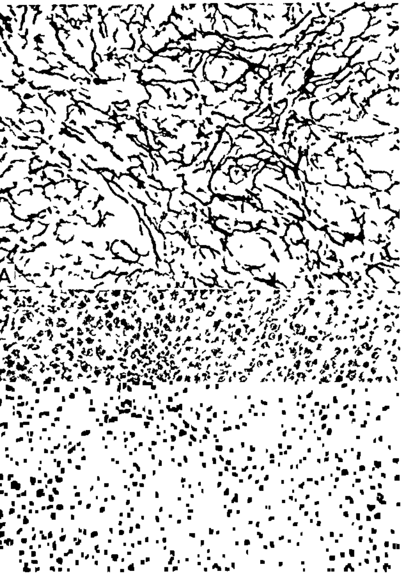


FIGURE 58

A Gomori stain Silver impregnation of rat liver shown in Figure 57 There is irregular new formation of reticulum fibers (x170)

B Hematoxylin eosin stain Liver of rat which had been on an 0.5% ethionine diet for 25 days There is severe liver cell damage and in the right upper corner necrosis around the central vein Interstitial infiltration composed of reticulo endothelial cells extends from the portal triad Surrounded by the infiltration an increased number of cholangioles is seen (x130)

tentatively as cholangioles In silver impregnations considerable new formation of reticulum fibers is noted (Figure 58A)

These lesions appear interesting for many reasons At this point, *I should like to emphasize their histogenesis based on observa* tions of different stages of the intoxication Dr Huerga has put such animals on the same diet for shorter intervals and after the initial stage of fatty liver a picture is found (Figure 58B) which is characterized by liver cell damage mostly localized in the center of the lobule, whereas from the portal triads cholangioles and in flammatory cells seem to stream into the lobular parenchyma With *higher power, near such cholangioles surrounded by inflammatory* exudate, a dilated vessel can be seen, which, following Elias, I assume to be a dilated arterial capillary We feel therefore that we have a lesion which combines diffuse hepatocellular damage, inflammatory changes, and a diffuse increased visibility or pro liferation of cholangioles Around the cholangioles, marked connec tive tissue proliferation is noted We feel that here is imitated to some degree, human hepatitis in which, *quite commonly, hepato*cellular damage is combined with cholangiolar changes, at least on the basis of functional alterations The cholangiolar changes referred to are in keeping with the studies of Watson and Hoffbauer, i e, interference with *bile flow*, elevation of *serum cholesterol* and alkaline phosphatase It is interesting that if these rats after one month on the ethionine diet are put back on a normal diet, both the hepatocellular as well as the cholangiolar changes disappear within approximately ten days I am not quite sure to what degree we are dealing with cholangiolar proliferation or increased visibility of pre existing cholangioles *In the initial stages, probably increased visibility is more important*

I should like to emphasize once more that Dr Elias' studies have stimulated me to look for cholangiolitic changes not only in and around the portal triads on the periphery of the lobules but also 2B within the lobular parenchyma itself *We have seen in a few human* cases cholangiolar changes within the lobular parenchyma and not only on the periphery

Watson You mean you have seen a picture like this in the lobules in hepatitis?

Popper Yes, sir We have seen it in cases of human hepatitis without liver cell damage but with high alkaline phosphatase high *cholesterol and severe jaundice fulfilling more or less the criteria* which you have set up

Smetana Can you prove that these are cholangioles?

Popper I believe so

Kniseli What are the specific characteristics of one cholangiole?

Popper Cholangioles have the following characteristics They are lined by low cuboidal epithelium which reveals hardly any basophilia of the cytoplasm They are often found in the vicinity of small but relatively thick walled vessels which Dr Elias considers arterial capillaries

Smetana Do they contain bile?

Popper In human cases they do contain bile

Smetana Where? Can you show that to me?

Popper I cannot show it in all specimens but in quite a few of the cases of what may clinically be called cholangiolar hepatitis bile casts can be seen within these little tubules which are not bile capillaries but still are located within the lobular parenchyma

Hartroft Would it not be possible by injection methods in the animal to demonstrate the continuity of these abnormal passages with the main biliary tree?

Popper Dr Smetana suggested previously an experiment which we are now planning to do namely to ligate the common duct in the ethionine rats to prove that what we are considering proliferated cholangioles contains bile

Hartroft One could allow India ink to flow into the bile duct in a retrograde direction

Popper Maybe we should do that

Grafflin We have tried with a dilute aqueous solution of fluorescein to obtain a retrograde injection of the bile canaliculi We have failed in the rabbit rat and mouse The ease with which the canaliculi break presumably at or near their junctions with the so called canals of Hering is extraordinary Although we have had no success I believe that some of our predecessors in the last century succeeded I am not at all sure from looking at Dr Elias pictures that he was successful with indigo carmine

Elias I got dilation of the bile canaliculi when injecting a dye into the main hepatic duct of a guinea pig but no breakage And I also got a retrograde injection of the bile canaliculi in an opossum

Knisely We have done one experiment many times in frogs which shows the connections of the bile canaliculi of the frog hepatic cell cord with the interlobular bile duct The results of the experiment are beautiful to see I had not known that the connection of the bile canaliculus with the interlobular bile duct presented a scientific problem so I have never been concerned about describ

Elias Dr Ralph (47) has shown dilated bile canaliculi after ligation, demonstrated with the phase microscope

Hartroft The problem of which I shall speak also involves a consideration of certain aspects of the structure of the liver It is now agreed by most investigators who have studied the early stages of experimental dietary cirrhosis produced in choline deficient rats (48,49) that the initial sites of fibrosis are around the radicles of the hepatic vein However, in the livers of rats that have been maintained on a low choline diet for a sufficiently long period (six or more months), until the cirrhosis has reached a stage of gross nodularity, *fibrosis can be demonstrated around those portal triads* which are large enough to be easily visualized under the low magnifications of the microscope Evidence has been already presented (14) which indicates that the manifestations of choline deficiency in livers of rats develop initially in nonportal regions and are always most advanced in these sites Why then should liver parenchyma adjacent to large portal triads become involved by fibrosis in later stages of this type of cirrhosis In the first pub-

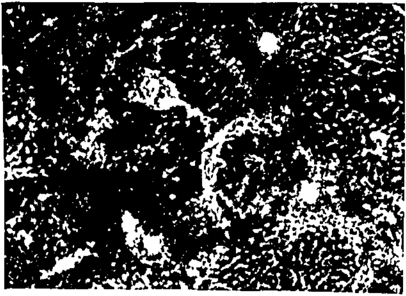


FIGURE 59 A conducting portal vein running upward in the center of the field has been cut longitudinally Parenchyma adjacent to it has in some regions become necrotic Above and to its left is a terminal venule surrounded by parenchyma which has escaped necrosis Frozen section stained for fat Ischemic liver of a dog (x200)

lished descriptions of experimental dietary cirrhosis (50), this type of periportal lesion was noted The data to follow will attempt to provide an answer to these questions

In Figure 59 is shown a microscopic field under low magnification of the liver of a dog subjected to ligation of the hepatic artery and the establishment of an Eck fistula by Dr Rappaport The necrosis which developed is nonportal Around the terminal branches of the portal vein, the hepatic parenchyma has been spared presumably because these liver cells have first access to whatever blood is still reaching the organ after the operative procedures But around the large branches of the portal vein the necrosis has crept up to the adjacent parenchyma, even though this tissue is geographically periportal This suggests that parenchyma in the latter position is not so favorably placed with reference to blood supply as that which surrounds the terminal venules of the portal vein

Figure 60 shows a thick (100μ) cleared section of the liver of a rat, which had been injected with India ink at the time of sacrifice The injection media was introduced via the left ventricle under



FIGURE 60 A terminal portal venule on the right is shown as it distributes blood to the surrounding sinusoids The central vein (center of field) is surrounded by swollen cells which have narrowed and separated the *intervening sinusoids* (x300)

physiologic pressure, so that all the patent vessels of the liver were filled This rat had been given a minimal dose of carbon tetrachloride twenty-four hours before it was killed The resultant parenchymal swelling around the radicles of the hepatic vein permit identification of the centrolobular regions in the photomicrograph, because here the sinusoids are separated from one another and narrowed The terminal branches of the portal vein may be seen as they link with sinusoids in a manner resembling the junctions between the branch of a tree and its terminal twigs In the liver, these terminal branches are venules, not veins

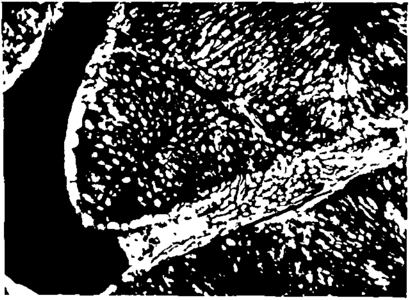


Figure 61 [illegible]

[illegible] in a geographic sense (×300)

In Figure 61 are shown vessels which Dr Elias has aptly termed the large "conducting veins" The clear space of which Dr Elias spoke is well demonstrated around these large veins This area is clear, I believe, because direct communications between the sinu-

soids and this large conducting vein are absent, or at best very rare The section illustrated is a thick (100μ) cleared slice of liver, with the lower plane of section cut through the large portal vein so that *its contained ink dropped out* This is fortunate, for where the vein is still filled with ink one sees apparent communications between it and the immediately adjacent sinusoids They are not actually communications, for these sinusoids do not run into, but over, the vein, as can be clearly seen where the ink has dropped out of the vessel Thus, most of the blood reaching liver cells adjacent to this large vein has to go first into the terminal or distributing portal venule and then pass along sinusoids to reach the parenchyma adjacent to the large conducting vein Although parenchyma in such a position is of course, geographically periportal it is actually nonportal if we are speaking in terms of blood flow and the order in which liver cells receive fresh blood Tissue next to these large conducting veins does not receive blood until the cells around the terminal venules have been supplied This provides an explanation for the presence of necrosis around the large conducting portal veins and its absence around the terminal venules The ischemic necrosis produced by Dr Rappaport's interference with the blood supply of the liver is essentially nonportal in its distribution Parenchyma around the large conducting veins becomes necrotic, for although it is periportal in a geographic sense, it is actually non portal in relation to the blood supply of each liver lobule and non portal in a functional sense

I should now like to refer to Figure 53 in Dr Rappaport's presentation, which he borrowed from the series I am presenting The fibrosis (clear areas) in this thick ($100\ \mu$) cleared section of the liver of a choline deficient rat injected with India ink is still in an early stage of development The trabeculae surround the central veins (at the edges of the field) and link them together in an annular fashion The distribution of the fibrous tissue illustrated follows the principle enunciated earlier, that lesions due *to choline deficiency develop first in the most nonportal areas of* the liver then in the next most nonportal regions, and so on The most nonportal regions are of course those surrounding the central veins and it is here that the fibrous tissue first develops (51) The next most nonportal regions are those which link together adjacent central veins for if one draws a line in any other direction, one is proceeding more directly toward portal areas Thus, the fibrous tissue extends from one central vein to the next, linking them together

In the photograph, however, it is apparent that fibrous bands are also growing from the annular ring toward the vessel in the center of the field, which is a large conducting branch of the portal vein In so doing, the fibrosis pursues a path midway between the terminal portal venules (distributing vessels) which fan out like spokes of a wheel from the large portal vein that forms their hub A consideration of the spatial relationships demonstrated in the Figure reveals that again the lesions are following the principle that the most nonportal regions are always affected first Notice that the trabeculae extending toward the conducting portal vein actually have made contact with its adventitia in a few places even at this comparatively early stage in the development of the cirrhotic lesion The fibrosis is becoming geographically periportal, although *in a functional sense, only nonportal parenchyma has been involved* After leaving one of the small terminal portal venules, blood must traverse just as many sinusoids (and pass just as many liver cells) to reach cells beside this large portal vein, as it would to reach other cells near the central veins

Figure 62 illustrates an ink-injected, cleared, thick slice of the liver of a choline deficient rat in a considerably more advanced stage of cirrhosis than that shown in Figure 53 The conducting portal veins running upward toward the surface of the organ have been cut longitudinally instead of in cross section as in Figure 61 Several stages in the development of the lesion are shown *in this* single field In the upper left can be seen a group of sinusoids which almost obscure their afferent terminal venule which distributes entering blood to them Here there has been little replace*ment of parenchyma by fibrosis, and thus almost all of the liver here* contains injection media The terminal venules below this, however, supply groups of sinusoids which have a "withered" appearance, for together they make up only small trees or bushes at the end of each venule, and they are surrounded by clear noninjected areas where *the sinusoids have* been replaced by fibrotic tissue Farther down this large conducting branch of the portal vein, the fibrosis around the laterally branching, terminal venules becomes *more* advanced The trabeculae actually encircle each terminal venule and extend to the root of the venule where it leaves the conducting vein Thus at such points, the conducting vein is surrounded by fibrosis If this portion of liver shown in the Figure had been sectioned at right angles to the path of the large conducting veins (instead of parallel with them as shown), isolated portions *of these* large portal veins *would have appeared to be enmeshed in fibrous*



FIGURE 62 The pattern of the fibrosis in a section of a cirrhotic liver of a choline

tissue (If the section were a conventional one of only 5 or 6 micra in thickness, the large portal triads, which would stand out very clearly under the low magnifications of the microscope, would be seen surrounded by fibrous tissue If one were attempting to deter mine on this basis alone whether the fibrosis was periportal or nonportal, it would be natural to call this a portal type of cirrhosis because of the preserved fibrosis around these prominent portal triads The fibrosis at this stage is periportal, but only in a geo graphic sense

Actually, if the observer were to search in the section for a portion that includes the terminal portal venules, he would find that the surrounding parenchyma was free of fibrosis Thus, he would know that from a functional standpoint the fibrosis is actually non-portal Terminal portal venules are frequently not included, how ever, in the plane of section, owing to their small size When they are present, they are inconspicuous for they are little larger than the sinusoids But, using the higher magnifications of the micro scope, one can always find and identify them for they are accompanied by small biliary ductules The significance of these points will be discussed further

Figures 63 and 64 show progressively later stages of cirrhosis in thick cleared slices of the livers of choline deficient rats that have been injected with India ink In Figure 63, the encircling trabeculae are frequently in the form of a heart shaped outline with the indentations at the sites of origin of the terminal portal venules, where they arise from the parent, conducting portal vein In Figure 64, it can be noted that every large vessel whether it be part of the portal or of the hepatic system of veins, is surrounded by fibrous tissue In the region of the small terminal portal venules however, the parenchyma is preserved showing that the lesion, from a functional viewpoint, is essentially nonportal in distribution.

Smetana Where are the bile ducts?

Hartroft They cannot be visualized in this type of preparation as these are thick slices cleared in benzyl benzoate The bile ducts are transparent and invisible

Figure 65 is a conventional microsection stained to demonstrate connective tissue from the same liver illustrated in Figure 64 Every large vessel is either surrounded by or adjacent to fibrous tissue Large portal triads are so involved that if inspection of such a section is confined to the low magnifications of the microscope by which terminal portal venules cannot be easily identified one would conclude that this was a portal or periportal fibrosis



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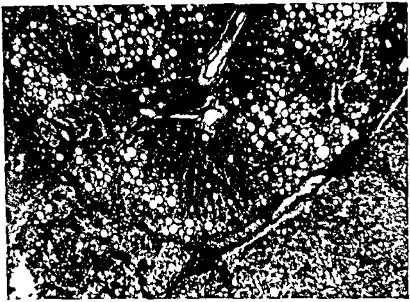


FIGURE 65 Paraffin section of cirrhotic liver of a choline deficient rat McGregor connective tissue stain There is an increase in connective tissue around the large veins (×300)

Under higher magnification (Figure 66) the geographic type of fibrosis around large portal triads is again demonstrated The central portion of the photomicrograph is enlarged in the inset A small bile ductule and vessel are present and the latter I have interpreted as a terminal portal venule The surrounding parenchyma is free of fibrosis indicating that the distribution from a functional viewpoint is nonportal

Figure 67 is from a case of alcoholic cirrhosis in man It demonstrates the same features shown for the rat in the previous illustration A large portal triad containing a conducting portal vein is surrounded by fibrous tissue The upper and central portion of the field is enlarged in the inset to show a small set of vessels surrounded by nonfibrotic parenchyma Again I have interpreted this as a terminal venule of the portal system of veins If it is one of the arterioles of which Dr Elias has been speaking it is still a vessel bringing fresh blood to the immediately adjacent parenchyma and as the latter is free of fibrosis it would still indicate that the distribution of the fibrotic lesions in this human alcoholic is functionally a nonportal one this despite the fact that geographically



FIGURE 66 Paraffin section of cirrhotic liver of a choline deficient rat McGregor connective tissue stain This is from a later stage in the cirrhosis than shown in Figure 65 (x100 Inset x800)

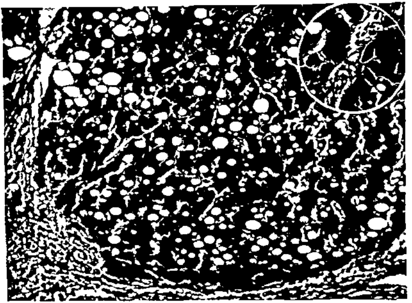
periportal tissue around the large conducting veins has been replaced by fibrous tissue)

Knisely Let us hear a list of the specific criteria upon which one would make a decision that a given visible vessel is an arteriole and nothing else

Hartroft That is exactly the question I should like to ask Dr Elias Would he tell us how he identifies these small vessels as arterioles They are very small and their walls are of simple construction I would never have suspected them of being arterial or arteriolar in nature They fulfill the criteria by which I identify venules and they correspond in their position to the small terminal branches of the portal vein present in a thick cleared slice where their sites of origin from large conducting veins make their venous nature obvious

I am perfectly willing to re examine these vessels in the light of any criteria suggested by Dr Elias which will aid in identifying whether they are of arterial or of venous origin

Whatever these vessels may eventually prove to be they still serve to identify the surrounding tissue as that which first receives



Toronto)

fresh blood entering the liver lobule This tissue is then periportal from a functional viewpoint, as well as in a geographic sense, in contrast to the large conducting veins which are surrounded by tissue which is periportal only in a geographic regard Thus, the presence or absence of fibrosis around terminal portal venules is the cardinal diagnostic feature in the attempt to determine whether a lesion is functionally periportal or nonportal Fibrosis around large conducting veins indicates only that the lesion is extensive and does not afford a clue to its functional pathogenesis If one accepts this, I believe it affords an explanation for the finding of Goldblatt and others that in the advanced stages of experimental dietary cirrhosis in rats the cirrhosis is portal Yet, we know from studies of the early stages of the lesion that it is a nonportal type of involvement and spreads from the most nonportal areas to those closer to the portal regions in a progressive manner Further, the last two Figures demonstrate that although the cirrhosis in the rat has been termed nonportal, while that in alcoholics has always

been regarded as portal in actual fact the distribution of the fibrous tissue is essentially the same in that parenchyma around the functional portal areas (supplied by terminal venules of the portal veins) is spared in both types In deciding whether a lesion (whether it be fibrosis necrosis or neoplasia) is portal or non portal in its distribution the small terminal portal venules should be examined and the diagnosis based on the presence or absence of the pathologic changes in the surrounding parenchyma We suggest that large conducting veins do not offer a reliable guide for this purpose for the reasons presented Clinical pathologists might well find it rewarding to re examine lesions in livers of man with this in mind for such a study might assist in clearing up certain confusing points now existent in the literature of hepatic pathology

Smetana Are these persisting vessels or could it be that they are regenerated vessels?

Hartroft They are persisting vessels or else they would not be in the center of nonfibrotic areas where they supply intact sinusoids

Smetana Do we have to think of this as a static lesion or a dynamic one?

Hartroft I think one can quite certainly regard these small terminal portal venules as ones which were present in the liver before the onset of the pathologic lesions for even in the few slides I have shown here they can be identified and traced at all stages in the development of the lesion from its initiation to its final appearance of grossly nodular cirrhosis

Smetana Even if the entire liver structure is changing you would assume that the vascular structure remains stationary?

Hartroft I am assuming that in the regions which are not affected by the lesions the sinusoids and the hepatic architecture would remain essentially the same

Smetana Why do you assume they are not affected? Most of us believe that this is a regenerated part of the liver and that it has been thoroughly affected

Hartroft Yes I have been speaking entirely of destructive lesions which do not involve areas around terminal portal venules Regeneration of course proceeds in such regions but this would be accompanied chiefly I should think by growth of the sinusoids supplied by these terminal venules rather than by appreciable growth of the venules themselves

Best Dr Elias have you some other slides to show?

Elias To answer Dr Hartroft's question concerning the arterial capillary and a sinusoid, if you have these together there can be no mistake Arterial capillaries are circular in cross section because they are under high pressure They are small very frequently smaller than the nuclei of the liver cells

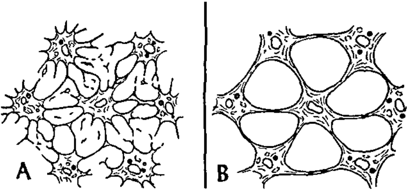


FIGURE 68 Diagrams illustrating the division of a lobule into several cirrhotic nodules and the elimination of the central vein from the parenchyma

However, I want to raise a question Figure 68 is a diagram of the development of the cirrhosis in the case of combined periportal inflammation and very slight, prolonged congestion, as Dr Popper and I have worked it out Figure 68A shows a liver lobule. Throughout the liver lobule, micromembranes arise

Tullis There is no such thing as a liver lobule, is there?

Elias Yes, under normal blood pressure conditions there is a liver lobule

Rappaport What kind?

Elias This one is a hepatic lobule The congestion in this case was very mild so mild that no lobular inversion has occurred

When these membranes aggregate to form septa (Figure 68B), they cut up the lobule like a pie and each of these nodules becomes what we call a primary nodule or nodule of the first order The former central vein has been eliminated from the parenchyma

If a slide shows a nodule and connective tissue around it and a large vessel in the connective tissue, that does not have to be a large conducting portal vein because it is outside the parenchyma But hepatic veins (central veins) have been eliminated from the parenchyma

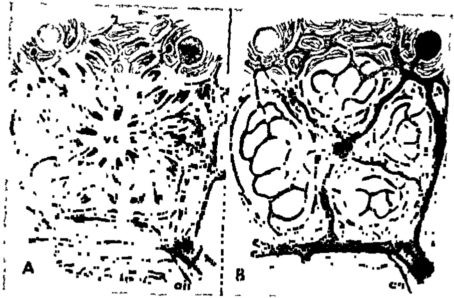


FIGURE 69 Diagram illustrating the origin of porto-hepatic shunts during the division of a lobule into several nodules, in cirrhosis ah hepatic artery, S in Figure A sinusoids S in Figure B porto hepatic shunt that originated from a sinusoid by inclusion into a septum, VC central vein VP portal veins The light areas in Figure B are the nodules

At A in Figure 69, there is a lobule The black lines indicate developing *micromembranes* These *micromembranes* aggregate to form septa, as can be seen at B Sinusoids become incorporated into the septa and, being deprived of a parenchymal environment, they become veins These former sinusoids then shunt the blood from the portal to the formerly central vein The vessel in the center of Figure 69B is not a large conducting portal vein but a former central vein However, most of the blood now bypasses the parenchyma and only a little goes into the parenchyma

The arterioles are of very great importance for the maintenance of the nodular parenchyma

Knisely Let me interrupt here You are demonstrating a beautiful hypothesis, but Dr Rappaport showed that exists in the healthy liver, which is in strict agreement with Mall's papers

Elias What I am showing is a pathologic specimen based on many reconstructions This is only the synthesis of many observations and not a hypothesis I also have many slides to support Dr Rappaport's report

The existence of these interlobular arterioles is important because those which happen to lie in one of the cut-out pie pieces will still

supply enough blood to the nodule to keep it alive These are the little vessels which Dr Hartroft thought at first to be very small portal branches, but in effect they are arterioles

Hartroft Would you tell us how you identify the arteriolar nature of these small terminal vessels?

Elias Figures 23 and 24 of my 1949 paper (21) illustrate the point well The arterial capillaries are narrow and round due to high pressure, while the sinusoids are larger, frequently collapsed

Knisely Both arterioles and sinusoids will change from either diameter to the other while you watch them Each can and do change in size and in shape, in a matter of minutes

Elias I admit they must change

Knisely Then you cannot define them separately because of their sizes

Elias I can also define them because they are located in a very tender trabeculae of connective tissue which encloses both the cholangiole and the arteriole They are the same thing which Wakim and Mann (52) have called "arterial sinusoids" Sometimes they are larger depending on the state Wakim and Mann have shown that

Knisely But this is an evasion If you say that diameter is a criterion by which you can identify a vessel as an arteriole, then you will have to use the criterion consistently You cannot, in one case, say you distinguish them by size, and in the next moment say differently Your forms of logic don't stand up

Elias You are right Size is not the only criterion, but an important criterion is the roundness, which shows arterial pressure in the one vessel, and the collapsed state —

Knisely What shape would you think a given vessel would be if cut in another direction?

Elias Which one?

Knisely A sinusoid if it were cut exactly at right angles to its long axis If cut exactly crosswise, it would be circular

Elias Not necessarily, because the human liver is a sacculosinusoidal liver

Knisely So far I have not seen any valid evidence presented that there are different types of saccular and tubular livers The things that have been shown might easily be differences in physiologic state rather than defined morphologic differences as permanent parts of the architecture That is a weak point in the argument for the moment at least

Hartroft Dr Elias how do you determine whether the vessel that you previously referred to is venular or arteriolar in nature?

Elias First of all because these things have been traced in serial sections to the arteries. Secondly they are always accompanied by a cholangiole The cholangiole need not necessarily be accompanied by an arteriole, but wherever there is an arteriole, there is also a cholangiole and it is always in a trabecula together with it

Hartroft Is there no histologic criterion to be found in the wall of the vessel itself?

Elias No, these have only endothelial lining, no muscular lining In fact many of the arterial vessels in the portal canal are only lined by endothelium as Weatherford (53) has shown

Knisely Dr Elias you showed one slide before (Figure 37) in which you raised the point of arterioles going clear across the lobule to the central vein

Elias No they don t

Knisely You said they gave off branches all the way along You have an injected specimen which shows a definite rather straight longitudinal vessel going quite a long way

There are in the history of the study of the structure of the liver lobule a number of questions about the distribution of the terminal branches of the hepatic artery The artery as you know contains arterial blood with the same saturation of oxygen and of other anabolites as does each and every other artery of the body The *portal vein ordinarily has a much lower concentration of oxygen* than does arterial blood Hence the anatomic distribution of the terminal branches of the hepatic artery are most important because *they definitely limit the histologic positions to which highly oxygenated* arterial blood can possibly be carried within the living liver

There are two major classifications of ideas concerning the terminal distribution of the branches of the hepatic artery One is as follows The terminals of the hepatic arteries give branches *to capillary networks which are located* (*a*) in the walls of the portal vein (*b*) in the walls of the hepatic artery itself (*c*) in and around the walls of bile ducts (*d*) in the walls of the hepatic nerves Sometimes branches of the arteries are thought also to cross the lobules and supply specific capillary networks which are the vaso vasorum of the walls of the veins which drain the liver that is the central veins the sublobular veins and larger hepatic veins A reference to this group of artery branches occurs in H Braus (54)

According to one set of ideas all of the hepatic artery blood
terminal branches
networks where it
has become second
hand blood does it pass into sinusoids of the liver lobules Accordingly branches described above have been termed the internal roots of the portal vein" According to this set of ideas the liver sinusoids receive secondhand venous blood only Aunap 1932 was one of the last protagonists of this set of concepts of the distribution of the terminal branches of the hepatic artery

The next set of ideas is that in addition to all the terminal branches of the hepatic artery named above there are large numbers of branches of hepatic arteries which directly join and conduct blood to the hepatic sinusoids Mall 1906 found branches of this latter category So did Olds and Stafford 1932 Wakim and Mann found these hepatic artery branches in some of the radial segments of lobules and failed to find them in other radial segments Wakim and Mann published their failure to find the terminals as though they had found the terminals absent This is an obvious selective interpretation of negative evidence Knisely Bloch and Warner in "Selective Phagocytosis (6) found that every sinusoid or almost every sinusoid received a branch which they named "the arterial sinous twig" from the hepatic artery This means that it is anatomically possible for arterial blood to be distributed to every sinusoid in the liver of frogs The same has also been seen in the Rhesus monkey Obviously a similar search should be made within the livers of many other species

The next question is where they join the sinusoids According to one set of ideas the terminal arterioles join the sinusoids a quarter to a third of the distance from the portal vein across to the central vein This has more adherents and I think a little better evidence There are not more than three or four papers in the literature in which people have thought that they could trace an artery clear across close to the central vein before it joins the sinusoids

If I remember correctly Dr Elias presented Figure 37 saying Here is an artery going clear across before it joins the sinusoids so that the sinusoids it might be said would not have gotten much oxygen by lateral diffusion

An injected vessel is seen in Dr Elias slide However before one can say the artery goes clear across the lobule before it joins the sinusoid there must be a method a good method of identify

ing and separating the artery from the sinusoid What criterion do you use to know where the end of the artery is, and where is the beginning of the sinusoid which it joins?

Elias First, I never said nor probably shall ever say, that this goes to the central vein, because the central vein is located at some distance above the upper margin of the picture

Knisely Or in the neighborhood of it I think what you actually said is that it gave off branches all the way across

Elias *My original idea was wrong because it was based on the* fallacious experiment after Chrzonszczewsky I have thrown that all out and made these new injections Please forget what I said a few years ago, because it was arrived at with wrong evidence The points of junction with the sinusoids which appear as large, black masses can easily be seen in Figure 37

Knisely Where is the end of the artery and the beginning of the sinusoid?

Elias The end of the artery and the point where it empties into the sinusoid is at the point where the thin lines join the large black spots and stripes

Knisely How do you know?

Elias This large space is no longer an arteriole It is a sinusoid

Knisely *You don t think that is a link?*

Elias The arteriole or arterial capillary opens at this point into this network of sinusoids These points are clearly visible in Figures 36 and 37

Rappaport May I ask where the central vein is?

Elias The central vein is quite a distance above the upper margin of the picture (Figure 37)

Rappaport Then the arterial capillaries still open into sinusoids which are close to the terminal portal branch

Knisely It is close to the artery

Elias The longest distance traversed by intralobular arterioles or arterial capillaries is approximately two thirds the distance be tween portal canal and central vein I don't think they go any farther

Knisely For the record I should like to describe the criterion which we have always used to identify the point at which a ter minal arteriole sinuous twig joins the hepatic sinusoid As one looks at such a junction in a healthy liver it is not possible to tell the exact point at which the artery ends However, the artery is lined with vascular endothelium which under ordinary conditions is not phagocytic and the sinusoid is lined by a membrane every

part of which is highly phagocytic It consists of a tubular sheet of so called Kupffer cells Thus, to find the end of the artery and the beginning of the sinusoid in a living preparation, one injects a very small amount of India ink into the animal's general circulation Each particle of the ink receives a coating, probably a protein from the blood plasma, and is carried downstream toward the liver As particles pass into the hepatic artery, they do not stick at any point to the lining of the artery, for that lining is ordinary endothelium But whenever a particle passes from the part of the tube lined with endothelium into a part lined with phagocytic cells, the particle is suddenly ingested by a cell which is a part of the wall of the tube Thus, the act of phagocytosis marks the position of the beginning of the sinusoid And in our experiments the position at which the artery joins the sinusoid has not been farther than one third of the radial distance from the periphery of the lobule to its center The position of the junction is usually between one fourth and one third of the radial distance to the center of the lobule

There are reports in the literature that the artery goes clear across and joins the sinusoids in the neighborhood of the central vein, and I suspect at present that that is due to cutting sections at the end of the whole lobule and assuming they were cut across the middle of the lobule

Waterlow In material I studied in Africa there seemed to be some special property of the sinusoids running between neighboring tracts For instance, they tend to be particularly wide and malarial pigment tends to congregate in them Also, as fibrosis develops in these livers, the walls of these sinusoids seem to have particularly thick fibers in them I wonder whether you can explain that because that is one place where presumably these intralobular capillaries and bile ducts don t run Secondly, can you say anything about the nature of this micromembranosis that develops in the walls of the sinusoids?

Elias I am on thin ice here, especially since I have not yet been able to convince my co worker, Dr Popper, of my idea Tentatively, however I have the following idea In these pathologic conditions, the permeability of the sinusoidal wall — the reticulo endothelial wall — is increased and some material seeps through This material condenses on the outside of the sinusoid, that is, in the tissue spaces

Figure 70 illustrates this hypothesis It also illustrates how I came to these strange ideas which Dr Popper does not share at least, he has not committed himself Here is shown an enlarged sinusoid sur

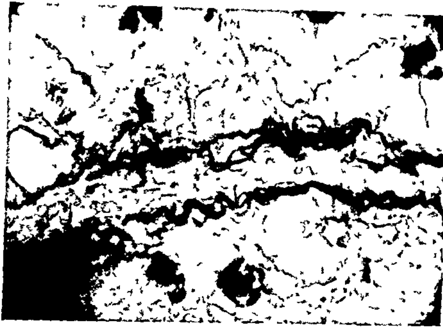


FIGURE 70 Micromembranes aggregating to form membranes and a septum in a very early stage of development An enlarged sinusoid is seen just incorporated into this young septum about to become a porto hepatic shunt (internal Eck fistula)

rounded by many membranes and one sees many layers of mem branes I assume that from this sinusoid the material has been secreted or has penetrated through the wall I don't know why, but this material has hardened has collagenized on the outside, and the first one that has been given off is the outermost micromem brane Then after a little while another one was secreted in the same manner and hardened and this is perhaps a way in which this micromembranosis can develop

Dr Waterlow I don t know if I should impose upon you but I should be very pleased if you used the word syndesmosis rather than fibrosis to describe any kind of connective tissue, without committing yourself as to its structure

Waterlow Yes I should agree to that because I actually mean reticulin fibers You did not answer my question about the sinusoids between neighboring portal tracts At any rate in people with malaria the pigment accumulates so that it forms a ring connecting neighboring portal tracts This syndesmosis too seems to develop more or less along the same channels I don t quite understand why that should be

Knisely The phagocytes which contain malarial pigment are exactly the same kind of cells which are known to migrate within the liver lobules after they have ingested India ink injected into the vascular system The migration of hepatic phagocytes after they have ingested material has been studied by Mortensen of the University of Wisconsin and by Wilson and his colleagues at Brown University

Hartroft Dr Elias, you referred to the vessel in Figure 70 as a sinusoid Might it not be a vein surrounded by its usual adventitial coat? Hasn't that a very large diameter for a sinusoid?

Elias It has, by all means This Figure is from a serial section, 100 serial sections were made It is just an enlarged portion of the sinusoidal network We have actually built a model of this series and it can be shown that this vessel runs from a portal space to the central vein It is not any longer a normal sinusoid but, as I said before, when many micromembranes accumulate and form a thick membrane and later thick membranes accumulate to form a septum, then sinusoids are included in it and are separated from the rest of the sinusoids These vessels which have been captured or incorporated become enlarged and assume the characteristics of little veins This, therefore, is a former sinusoid which is now a porto-hepatic shunt

Smetana What stain was used?

Elias Van Gieson

Best Is that the same sort of thing you are talking about, Dr Waterlow?

Waterlow It seems to be much more regular The reaction I saw around the portal tract was rather diffuse, instead of having a clean cut edge

Elias You are no doubt referring to a silver stain That shows reticular fibers and does not differentiate collagen

Best Dr Popper, do you have some remarks to make?

Popper I have promised Dr Elias to discuss briefly studies which we are conducting together concerning the histogenesis and the morphogenetic classification of cirrhosis These studies are based upon Elias three dimensional approach and his findings in the normal adult and embryonal liver

In principle, we feel there are three pathways along which cirrhosis can develop

1) The *postnecrotic type* follows the collapse of hepatic tissue after massive necrosis of several lobules This leads to an approximation of the portal and central canals and to stress in the surrounding

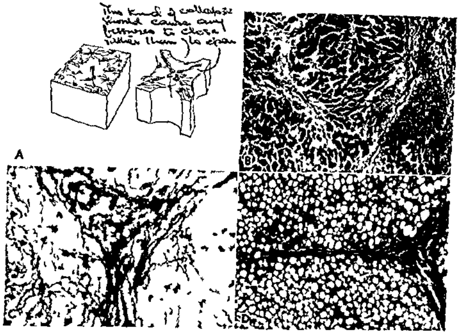


FIGURE 71

A Schematic drawing of massive necrosis of several liver lobules followed by collapse of the necrotic area and breaks in the surrounding noninvolved tissue

B Hematoxylin eosin stain Fissures in intact lobular parenchyma bordering on an area of recent postnecrotic collapse Liver cells are disappearing around dilated sinusoids in the fissures (x70) Reprinted by permission from Popper H Elias H and Petty D E Vascular pattern of the cirrhosis liver *Am J Clin Path* 22, 717 (1952)

C Van Gieson stain Micromembranes radiating from portal triad into lobular parenchyma in septal (Laennec s) cirrhosis (x210)

D Mallory stain Straight septum extending from portal trial in fatty cirrhosis (x90)

liver tissue not involved by necrosis (Figure 71A) This stress causes breaks of the tissue and in the developing fissures, liver cells disappear and sinusoids persist and dilate (Figure 71B) Membranes form within the fissures by collagen deposition around the pre existing reticulum Consequently, the lobules surrounding the *massive necrosis are also dissected by septa*

2) The *septal type of cirrhosis* (corresponding in general to Laennec s cirrhosis) results from primary formation of septa originating either from the portal or the central canals (*a*) In some forms of portal inflammation (e g, viral hepatitis) or irritation (e g, hemochromatosis) multiple fine membranes radiate diffusely from the portal canal into the lobular parenchyma (Figure 71C) These micromembranes subsequently aggregate into thicker two-dimensional sheets which as septa dissect the lobules (*b*) In fatty livers

similarly, micromembranes develop around shells of fatty cysts (Hartroft) and also around regenerative cells in the vicinity of these cysts It is, however, our impression at present that the main septa in the human fatty liver originate independently as straight sheets, often connected with the portal canals (Figure 71D) We feel that the alternation between irregularly distributed areas of fatty metamorphosis, regeneration, and normal tissue produces stress in the lobules due to uneven expansion, resulting in fissures (Figure 72A) In these fissures, circumscribed micromembranosis develops

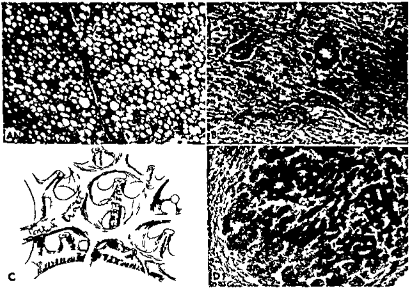


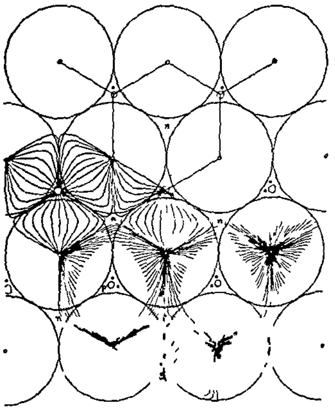
FIGURE 72

A Hematoxylin eosin stain Fissure in fatty cirrhosis apparently as a result of stress (x90)
B Van Gieson stain Cholangiolitic (trabecular) cirrhosis Trabeculae containing proliferated cholangioles are surrounded by fibers (x180)
C Schematic drawing depicting septa extending between the portal and central canals which cross in the three-dimensional space
D Mallory stain Cirrhotic nodule revealing several cell thick plates on its periphery ("blastema") and one cell thick plates in the center (x130)

into straight septa which may connect with micromembranes around the fatty cysts (c) Central origin of the septa results from the diffuse central micromembranosis in chronic passive congestion and (only rarely) as a sequela of centrolobular toxic necrosis Again aggregation of micromembranes causes the formation of the septa

Grafflin I simply want to say that I was very much interested n everything Dr Rappaport said For the record I regard it as mportant, since I don't think it is quite clear to many in this room hat he should state how his unit or lobule differs from that of Mall

Rappaport I have a slide from Mall's paper (24) which will llustrate the difference There is a basic difference between the icini I described and the portal unit which Mall described In



igure 74 the three triangular spaces around the portal canal are Mall's portal units I shall quote from his article The portal vein es of course (?*) in an interlobular space (?*), and its three

* The question marks are those of A M R

main branches extend into adjoining fissures Together they mark the center of the portal unit

It is evident from this quotation that Mall, by the description of a new static lobule, which is not well coordinated with function, admits the existence of two lobules (hexagonal and portal) opposing each other rigidly There is no other possible way to correlate the structure and function of a gland than to follow the natural course of the channels that supply the materials necessary for function and of those that carry away the products. These channels are the terminal afferent vascular branches and terminal bile ducts arising from a small portal field Each of these, when at first developed, supplied a definite amount of parenchyma which we call liver acinus'

Figure 75 is peculiar, but it will explain the psychological reason why for one hundred years we have stuck to this hexagonal stum

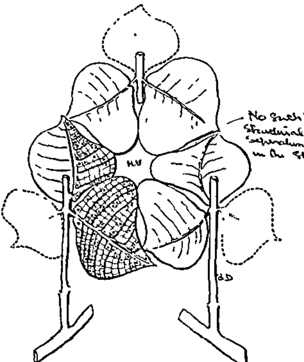


FIGURE 75 Three vertical branches corresponding to 3 bile ducts in 3 neighboring small portal spaces outline with the stems of their leaves an hexagonal field This will make the portal vein branches (which lie 3 the side of the bile ducts) to run parallel with the Hepatic Vein radicals, and they are said not to do this

45 FARBER, E, KOCH WESER, D, and POPPER, H The influence of sex and of testosterone upon fatty liver due to ethionine *Endocrinology* 48, *205 (1951)*

46 KOCH WESER, D, and POPPER, H Hepatic fibrosis produced by chronic ethionine feeding *Proc Soc Exper Biol & Med* 79, *34 (1952)*

47 RALPH, P H Observations on bile conducting surfaces in mammals (An abstract) *Anat Rec* 109, *339 (1951)*

48 ASHBURN, L L, *et al* The nonportal distribution of the trabeculae in dietary cirrhosis of rats and in carbon tetrachloride cirrhosis of rats and guinea pigs *Am J Path* 23, *159 (1947)*

49 GLYNN, L E, HIMSWORTH, H P, and LINDAN, O Experimental production and development of diffuse hepatic fibrosis ('portal cirrhosis') *Brit J Exper Path* 29, *1 (1948)*

50 GYORGY, P, and Goldblatt, H Hepatic injury on a nutritional basis in rats *J Exper Med* 70, *185 (1939)*

51 HARTROFT, W S The locus of the beginning of dietary cirrhosis *Liver Injury* Hoffbauer, F W, Editor Trans Eighth Conf New York, Josiah Macy, Jr Foundation, 1949 (p 126)

52 WAKIM K G, and MANN, F C The intrahepatic circulation of blood *Anat Rec* 82, *233 (1942)*

53 BREMER, J L *A Textbook of Histology* Rewritten by H L Weatherford, Philadelphia Blakiston Co, 1944

54 BRAUS, H *Anatomie des Menschen* Berlin Verlag von Julius Springer, 2nd ed, Vol II, 1934 (p 325)

55 POPPER, H, ELIAS, H, and PETTY, D E Vascular pattern of the cirrhotic liver *Am J Clin Path* 22, *717 (1952)*

56 UNGAR, H Transformation of the hepatic vasculature of rats following protracted experimental poisoning with carbon tetrachloride Its possible relation to the formation of urate calculi in the urinary tract *Am J Path* 27, *871 (1951)*

THE MECHANISM OF BILIARY EXCRETION IN MAMMALS*

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Most of us have been raised to believe that under normal physiologic conditions biliary excretion is an orderly process and this belief has been supported by most of the pictures we have seen of the bile canalicular system in livers prepared fixed and stained in diverse ways We are inclined to regard the bile canaliculi as delicate but definite channels which normally retain their tubular character unaltered save for modest generalized increase or decrease in caliber concomitant with variations in the degree of excretory activity The first successful visualization of the process *in vivo* by Ellinger and Hirt (1) who studied the excretion of fluorescein in the frog's liver by the technique of fluorescence microscopy brought striking confirmation of the views generally held and all doubts were temporarily stilled

There the matter might have rested had not the new technique of intravital fluorescence microscopy proved so appealing Hartoch (2) was the first to pursue the initial observations of Ellinger [illegible] d by poor [illegible] the other [illegible] of biliary excretion in both amphibia and mammals under normal and abnormal conditions and contributed much that was new As far as the normal process was concerned their observations were largely consistent with notions then prevalent and in retrospect it is to be regretted that they did not continue studies which had been so well begun

The picture changed suddenly with the brief announcement by Hirt (5) in 1934 of the results of an extensive investigation of

* This investigation has been supported by research funds given by Mrs William G Baker Jr The "Ultropak" microscope and accessories used have been made available on loan from the Armed Forces Institute of Pathology

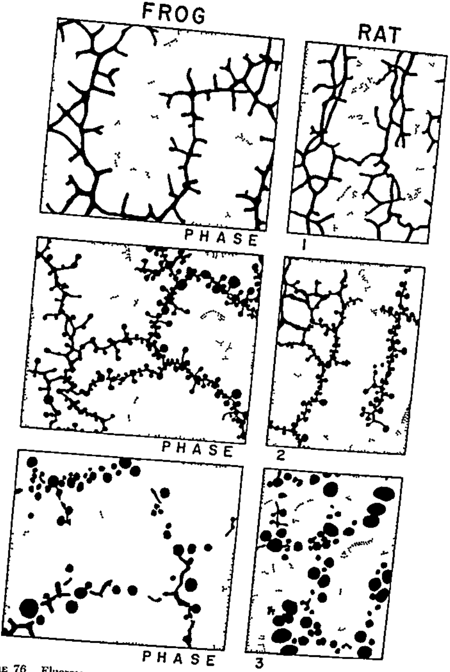
fluorescein excretion in the frog and rat undertaken in collaboration with Ansorge (6) and Markstahler (7) He described the normal process as characterized by extreme complexity, a comment for which students of the liver, working with other methods, could hardly have been prepared Speaking, as Hirt did with the full authority of one of the two individuals involved in the introduction and development of the technique of intravital fluorescence microscopy, it is not surprising that the problem was not further explored by others until a detailed report of their findings was issued by Hirt, Ansorge, and Markstahler in 1938 (8)

Stated briefly, they envisaged the normal process, in both frog and mammal, as comprising three successive phases, as follows (*a*) transfer of fluorescein by the hepatic cells to the bile canaliculi with complete delineation of the canalicular system in conventional patterns of sharply delineated tubular channels (*b*) an extraordinary "sprouting" of small fluorescein containing vacuoles from the canaliculi, (*c*) coalescence of the vacuoles and the development of discontinuity in the canaliculi to give a picture of widespread formation of intracellular vacuoles, from which the dye is slowly eliminated These three phases are illustrated in Figure 76

Such behavior on the part of the liver and of the bile canaliculi under normal conditions, could only be regarded as shocking by any serious student of hepatic structure and function Nevertheless the report of Hirt, *et al* was presented in a convincing manner, with seemingly adequate control studies and realization of possible pitfalls attributable to the methods employed Furthermore, all aspects of the work were illustrated by an artist, August Vierling of such distinction and integrity that his signature alone was enough to guarantee that the investigators had accurately observed the bizarre phenomena which they described

The abandonment of preconceived notions and the acceptance of new phenomena are much more difficult for some individuals than for others A desire for personal acquaintance with the more striking findings of Hirt *et al* led to a detailed re investigation of fluorescein excretion by the frog's liver as observed *in vivo* with the fluorescence microscope (9) Some of the results of this study were presented before the Seventh Conference on Liver Injury (10), and may be summarized briefly as follows

1) After injection of fluorescein the dye appears promptly in the plasma then in the hepatic cells and the bile canaliculi With a dosage of 30 mg per Kg, injected into the dorsal lymph sac, the peak of excretion is reached in approximately one hour at about



IGURE 76 Fluoresce n excre

ed frog and rat
Markstahler H
lebenden Frosch
Ztsch f Anat

15° C At this time, all axial bile canaliculi and their intercellular branches are sharply delineated at high fluorescent intensity, and the cytoplasm of all hepatic cells is diffusely stained at low intensity The sequence of events from the peak of excretion to complete elimination from the liver is essentially a progressive diminution in fluorescent intensity of the dye to nondetectable levels, achieved first in the hepatic cells, then (much later) in the bile canaliculi As long as the bile canaliculi are delineated, they persist as a continuous system, without detectable interruptions in their course

2) The phenomena observed by Hirt, *et al*, in the later stages of excretion ('sprouting,' discontinuity of canaliculi, intracellular vacuole formation), and interpreted by them as normal, play no role whatsoever in the normal mechanism of fluorescein excretion However the entire range of these phenomena can be observed in [illegible]

production

3) The liver responds promptly, in the usual manner, to repeated injections of the dye administered at any stage of the excretory process, and a refractory period of at least several hours duration, as claimed by Hirt, *et al*, does not occur with respect to fluorescein excretion

The potential dangers of prolonged observation of the liver in anesthetized animals with the fluorescence microscope were clearly demonstrated, and the conclusion was inescapable that, in the frog at least Hirt Ansorge and Markstahler had largely concerned themselves with abnormal phenomena It seemed highly unlikely that their unusual findings would prove to be any more valid for the normal mechanism of biliary excretion in the mammal than in the frog However, this proof had yet to be obtained, and judgment was suspended

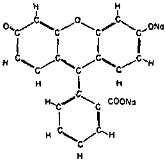
During the past year, we have had the opportunity of extending the earlier study to the mammal, using principally the white mouse and to a lesser extent the white rat We have employed a variety of fluorescent compounds fluorescein esculin and thioflavine S, all of which are rapidly transferred in high concentration to the bile canaliculi and can be sharply visualized under the fluorescence microscope It can be stated at once that the results of this study are what would be anticipated as far as the normal mechanism of biliary excretion is concerned The mechanism is fundamentally the same in the mammal as in the frog

Our equipment is simple consisting of a lightproof box housing an ultraviolet light source and the necessary filters and a Leitz "Ultropak microscope designed for work with incident light With our equipment as with most equipment utilized for fluorescence microscopy the term ultraviolet is used loosely since the beam includes some visible blue light The structure of the fluorescent compounds employed is given in Figure 77 which includes bromsulphalein for comparison One of these compounds thioflavine S exhibits such marked photosensitivity that the initially sharp pictures of the bile canaliculi are almost totally extinguished within a few minutes in any field held under observation While they persist however the pictures are highly informative and include many of the best we have seen

Our critical data upon the normal mechanism of biliary excretion in the mammal are derived from observations made as quickly as possible after sacrifice of animals in all stages of the excretory process The reasons for this are implicit in our earlier experience with the frog and have been amply confirmed by our recent studies We have been most discouraged by our attempts to apply the refinements of intravital fluorescence microscopy to the investigation of this problem in anesthetized mammals Under such conditions distortion of normal function would appear to be the rule rather than the exception So far as we could determine we had no alternative but to use freshly sacrificed animals and we are convinced that the information derived therefrom gives a picture of the normal excretory process which is valid in most important respects Ellinger is quite right when he states that the fluorescent image due to fluorescein changes very rapidly after death (11) However in our experience these changes are largely confined to the appearance of the cytoplasm of the hepatic cells and we have not observed any rapid or significant change in the pictures of the bile canalicular system in the immediate post mortem period

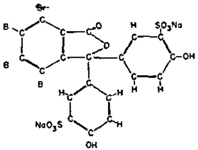
The general course of biliary excretion of fluorescein esculin and thioflavine S when administered to white mice in the same dosage and via the same route is summarized in Table XVI Fluorescein and esculin are eliminated rather rapidly and follow a similar course as far as time relationships are concerned With thioflavine S although the peak of excretion is quickly attained elimination proceeds far more slowly and traces of the dye may still linger in the canalicular system after twenty four hours With the dosage employed (30 mg per kg) esculin is routinely not detectable in the hepatic cells With fluorescein the cytoplasm is diffusely stained

FLUORESCEIN Na SALT (Uranine)



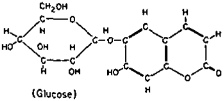
$C_{20}H_{10}O_5Na_2$ Mol Wt 376

BROMSULPHALEIN



$C_{20}H_8Br_4O_{10}S_2Na_2$ Mol Wt 838

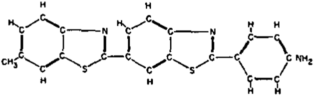
ESCULIN



(Glucose) (Esculetin)

$C_5H_{16}O_9$ $1/2\ H_2O$ Mol Wt <u>340</u>(367)

THIOFLAVINE S - A MIXTURE
PRINCIPALLY A METHYLATED AND SULPHONATED
DERIVATIVE OF PRIMULIN BASE



$C_2H_{15}N_3S_2$ Mol Wt 373

Figure

TABLE XVI

Observations Upon White Mice Sacrificed at Intervals Following Intraperitoneal Injection of Fluorescein, Esculin, and Thioflavine S 30 Mg per Kg

Interval	Intensity of Fluorescein in Bile Canaliculi				Interval	Intensity of Esculin in Bile Canaliculi			
	High	Moderate	Low	Very Low		High	Moderate	Low	Very Low
min					min				
5	—	Rare	3/4	1/4	5	—	1/8	7/8	—
10*	Most	Few	—	—	10	—	Rare	1/2	1/2
15	Few	Most	Few	—	15	1/5	2/5	2/5	—
20	Few	Most	Few	—	20	—	—	1/5	3/4
25	—	2/3	1/3	—	25	—	Rare	1/2	1/2
30	Few	Most	Few	—	30	—	—	Rare	All
45	—	Few	Most	Few	40	—	—	—	Few
60	—	Few	Most	Few	60	—	—	—	—
75	—	—	Rare	2/3	60	—	—	—	Few
90	—	—	—	—	80	—	—	—	—

* Fl orescein detectable n cytoplasm of I epatic cells

TABLE XVI (Continued)

Interval	INTENSITY OF THIOFLAVINE S: In Bile Canaliculi				In Hepatic Cells			
	High	Moderate	Low	Very Low	High	Moderate	Low	Very Low
min								
5	1/2	1/4	1/4	—	1/4	1/2	1/8	—
10	All	—	—	—	Rare	3/4	1/4	—
15	3/4	1/4	—	—	1/4	1/2	1/4	—
40	1/2	1/2	—	—	—	1/4	3/4	—
60	1/4	3/4	—	—	—	1/4	3/4	Few
90	—	7/8	1/8	—	—	—	3/4	1/4
hr								
3	—	Rare	3/4	1/4	—	—	1/4	3/4
7	—	—	All	—	—	—	—	—
12	—	—	—	All	—	—	—	—
24	—	—	—	1/4	—	—	—	—

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at and near the peak of excretion, and with thioflavine S, cytoplasmic staining persists for hours

Throughout these studies of the normal mechanism biliary excretion in mammals has revealed itself as a very orderly process, with none of the bizarre phenomena described by Hirt, *et al*, in the later stages of excretion The bile canaliculi are invariably visualized as sharply delimited, tubular channels, and to the very end of excretion they persist as a continuous system Compared with their delicacy in the early and late stages, they show an appreciable increase in caliber at the peak of excretion but are otherwise unaltered in appearance Segregation of the fluorescent compounds in vacuoles within the hepatic cells is never encountered

On the surface of the mammalian liver, we have regularly observed pictures which were new to us and may be unfamiliar to others The most superficial bile canaliculi usually appears as a polygonal pattern of varying extent and oriented in the plane of the liver surface Each of the polygons in the pattern delimits a single hepatic cell territory, and it soon became apparent that there frequently occurs in the mammal a more or less continuous subcapsular lamina of hepatic cells These laminae, which appear to be only one hepatic cell in thickness over the underlying sinusoids are continuous with subjacent laminae, which join them at approximately right angles and are interrupted at irregular intervals by varying numbers of sinusoids which achieve an immediately subcapsular position The structural relationships of these subcapsular laminae are quite similar to those of the "limiting laminae" surrounding the portal spaces as described by Remak (12) and Elias (13)

As is well known the basic architectural plan of the bile canalicular system in amphibia is one of mid axial canaliculi located in the center of the cords or laminae which are typically two hepatic cells in thickness and are provided with numerous radially projecting, blindly ending intercellular branches In mammals the situation is quite different and the three dimensional aspects of the problem have recently been analyzed by Elias (13) in considerable detail and in a highly contributory way With his conclusions (14 15) that the mammalian liver exhibits a laminar (and not a cord) type of organization and that the laminae are typically one hepatic cell in thickness we are in entire agreement Such arrangements can be observed regularly and clearly by our methods It is only in terms of the subcapsular laminae described above and of Elias concept of laminar structure that our pictures of the bile

canaliculi, as visualized at and just below the surface of the liver, can be satisfactorily interpreted

When completely delineated by fluorescein, esculin, or thioflavine S, the canaliculi in the surface polygonal pattern are everywhere continuous, either with each other or with canaliculi in the subjacent laminae Furthermore, such continuity can be repeatedly demonstrated as one focuses more and more deeply into the substance of the liver, to the maximum depth of penetration which is possible with our present equipment The pictures are so consistent and so convincing as to leave no doubt that the fundamental architecture of the bile canalicular system in mammals is that of a continuous never ending polygonal meshwork Using different methods, Elias (13) arrived essentially at the same conclusion several years ago, and similar views were held by a number of still earlier investigators We can only conclude from our own observations that so called "intercellular branches," ending blindly, must be of such infrequent occurrence in the normal mammal as to be insignificant

As visualized by fluorescence microscopy in the liver of the white mouse, the polygonal meshworks of the canalicular system exhibit a characteristic alternation in plane as one focuses into the substance of the liver In succeeding planes, the meshworks (and the associated hepatic laminae) are approximately at right angles to each other, and the pictures obtained confirm, in an almost diagrammatic and highly repetitive way, the essential correctness of the schematic stereogram of the saccular liver recently published by Elias (14) We are not ourselves artists, and up to now we have been loathe to involve others in the very difficult task of portraying accurately these complicated three dimensional patterns Figure 78 represents an admittedly crude and only moderately successful attempt to illustrate the observed relationships

Two additional aspects of the canalicular system merit brief comment at this time First in confirmation of the observations of Elias (13) we have encountered numerous instances of canaliculi coursing between the walls of sinusoids and adjacent hepatic laminae In the second place, in agreement with McIndoe (16) and others we would firmly deny the occurrence of *intra*cellular branches of the bile canaliculi under normal physiologic conditions Wachstein and Zak (17) are the most recent investigators to claim demonstration of such branches in the normal mammalian liver If we doubt the validity of their claim it is because we have observed the rapidity with which such "branches" can develop, in association

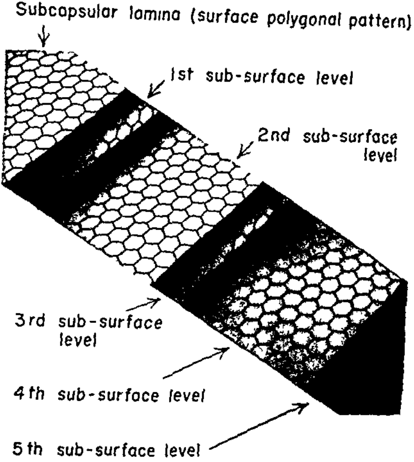


Figure 78 Diagrammatic representation of alternation in plane of laminar structure of mammalian liver

with other more obvious abnormalities in anesthetized animals and in post mortem material

Although we have made no particular study of the intrahepatic circulation by direct microscopic observation in anesthetized mammals our studies of freshly sacrificed animals offer strong indirect evidence that at least the great majority of the hepatic cells are normally accessible to an active sinusoidal circulation When fluorescein esculin or thioflavine S is injected intraperitoneally or intravenously complete delineation of the bile canalicular system is rapidly achieved as is uniform cytoplasmic staining by fluorescein or thioflavine S The consistency and speed with which such pictures are obtained focus attention once again upon the work of

Knisely (18,19,20) and others (21,22) upon regional and local variations and intermittence in intrahepatic blood flow The demonstration by Elias (14,15) that the hepatic laminae in mammals are typically one cell in thickness has a direct bearing upon this problem Since most of the laminae are presumably in contact with sinusoids on both surfaces, blood borne substances could still be offered equally to essentially all of the hepatic cells when approximately half of the sinusoids were inactive, provided this half were properly distributed throughout the liver substance

As mentioned earlier, Hirt, *et al's* (8) claim to have demonstrated a refractory period in the secretory activity of the liver could not be confirmed in the frog We have likewise failed to confirm it in the mammal As in the frog, the liver responds promptly, with complete delineation of the bile canaliculi, to repeated injections of fluorescein at any time during or following the excretion of dye administered by a previous injection There is no evidence for a refractory period of even brief duration, and Hirt, *et al's* observation of such refractoriness, extending over a period of several hours can only be attributed to grossly abnormal experimental conditions

In this account of the normal mechanism of biliary excretion, we have been compelled, however reluctantly, to refer more than once to inadequacies in the work of Hirt, Ansorge, and Markstahler (8) It is well to emphasize that their work was done with the best intentions in the world, at a great cost in time and effort, in the early days of the then new technique of intravital fluorescence microscopy At that time, the technique appeared to its originators Ellinger and Hirt (1,23), and perhaps even more so to many of their colleagues, to offer a most elegant method for the detailed microscopic investigation *in vivo* and at the highest magnifications, of many physiologic processes It is certainly unfortunate that Hirt, *et al*, became involved to such a large extent in abnormal phenomena, which they failed to recognize as such However, in the long run they have rendered a most important service to all students of liver function Their work serves as a warning of the gross distortion of normal hepatic function which can develop under experimental conditions which many competent, contemporary anatomists and physiologists would not only accept, but commend, as most suitable for investigation of the problem if it were being undertaken *for the first time* Furthermore, they appear to have observed the abnormal phenomena which they encountered with a high degree of accuracy, and they were responsible for having them illustrated in an outstanding manner Only those who have had personal ex-

perience with the fluorescent images obtained can fully appreciate the excellence of Vierling's drawings

In the mammal, as in the frog, we have observed the development of all of Hirt, *et al*'s aberrant phenomena (extensive sprouting" of small vacuoles from bile canaliculi widespread intracellular vacuole formation, discontinuity of bile canaliculi, etc), but only under conditions sufficiently abnormal to justify the conclusion that they have nothing whatsoever to do with the normal mechanism of biliary excretion They frequently develop in the livers of mice and rats maintained under anesthesia, and subjected to more or less prolonged observation under the fluorescence microscope They likewise appear with time in the livers of freshly sacrificed mammals, in the critical study of which we make it a practice to restrict observations to the first two minutes following initial visualization of the liver The entire range of these abnormal phenomena may be observed in the liver of a white mouse subjected to progressive anoxia for a period of ten minutes following injection of fluorescein then sacrificed and prepared for study as quickly as possible It is clear that neither anesthesia nor prolonged observation under the fluorescence microscope plays any essential role in their development This confirms our earlier experience with the frog (9), and in both vertebrate forms we are inclined to regard anoxia as the critical factor The pictures of more or less widespread intracellular vacuole formation, as observed in these fluorescence studies, would appear to be quite similar to those of so called watery" or 'anoxic" vacuolation of the liver, as described by numerous investigators under conditions of anoxia, decompression perfusion etc (24 25, 26 27,28,29)

A detailed account of our more recent studies upon biliary excretion in both amphibia and mammals appears in a recent issue of the *Bulletin of The Johns Hopkins Hospital* (30)

Knisely Are the nuclei visible in your preparations?

Grafflin In the frog, the nuclei can be readily visualized as non fluorescent areas In the mammal with our present equipment, the nuclei cannot be visualized

Smetana Are the bile canaliculi actually vessels or are they only potential spaces between and within the cells?

Grafflin By our methods we do not see any suggestion of the bile canaliculi in the normal uninjected, living or freshly sacrificed animal The question as to the true nature of the canaliculi is a complicated one and does not lend itself to brief discussion

Smetana We see them in cases of stasis of bile in pathologic conditions

Popper Do you employ any fixative in your preparations?

Grafflin No, none

Popper Are frozen sections used?

Grafflin In our freshly sacrificed animals, we expose the liver *in situ* and bring its surface under observation as quickly as possible

Shorr How do you kill your animal?

Grafflin Mice and small rats we sacrifice by crushing the cervical cord with a sturdy hemostat

Hoffbauer Could any of these dyes be used as a satisfactory test of liver function in mice?

Grafflin At the Seventh Conference on Liver Injury, Bradley reported that fluorescein is removed from the blood less rapidly than bromsulphalein both in the normal subject and in the presence of liver disease Also he reported that from 50 to 65 per cent of the dye is excreted in the urine Fluorescein would not appear to be particularly suitable for testing liver function and we do not have enough information along these lines about either esculin or thio flavine S

Popper Is it necessary to use a quartz optical system?

Grafflin No our optics are of optical glass, which shows good transmission of ultraviolet light in the range 360 to 400 millimicrons One would naturally want quartz only for efficient delivery of ultraviolet light to the object It is completely unnecessary for viewing the fluorescent images

REFERENCES

1 ELLINGER P and HIRT A Mikroskopische Untersuchungen an lebenden Organen Methodik Intravitalmikroskopie *Ztschr f d ges Anat* (Abt 1) 90, *791 (1929)*

2 HARTOCH W Zur Morphologie der Leber und Nierensekretion Lebendbeobachtungen im Luminescenzlicht *Ztschr f d ges exper Med* 79 *538 (1931)*

3 FRANKE K and SYLLA A Mikroskopische Lebendbeobach tungen innerer Organe Gallencapillar und Leberzellenstudien mit Mikrophotogrammen am lebenden Frosch *Ztschr f d ges exper Med* 89, *141 (1933)*

4 ——— Mikroskopische Legendbeobachtung innerer Organe Beobachtungen an der Leber gesunder und kranker Warmbluter *ibid* 93, *582 (1934)*

5 HIRT A Über mikroskopische Untersuchungen an der lebenden Leber bei Frosch und weisser Ratte *Verhandl d anat Gesellsch* 42, *222 (1934)*

6 Ansorge, I *Die Ausscheidung von Fluorescein und Trypaflavin durch die Leber des Winterfrosches* Inaug Diss, Heidelberg Coburg. Togeblatt Haus, 1933

7 Markstahler, H *Die Ausscheidung von Fluorescein und* [illegible]

8 [illegible]

9 Grafflin, A L The excretion of fluorescein by the liver under normal and abnormal conditions, observed *in vivo* with the fluorescence microscope *Am J Anat 81, 63 (1947)*

10 ——— *Studies of fluorescein excretion by the liver under* normal and abnormal conditions *Liver Injury* Hoffbauer, F W, Editor Trans Seventh Conf New York, Josiah Macy, Jr Foundation, 1948 (p 73)

11 Ellinger, P Fluorescence microscopy in biology *Biol Rev 15, 323 (1940)*

12 Remak, R *Untersuchungen über die Entwickelung der Wirbelthiere* Berlin, G Reimer, 1855 (p 116)

13 Elias, H A re examination of the structure of the mammalian liver II The hepatic lobule and its relation to the vascular and biliary systems *Am J Anat* 85, *379 (1949)*

14 ——— A re examination of the structure of the mammalian liver I Parenchymal architecture *ibid*, 85, *311 (1949)*

15 ——— The liver cord concept after one hundred years *Science* 110, *470 (1949)*

16 McIndoe, A H The structure and arrangement of the bile canaliculi *Arch Path 6, 598 (1928)*

17 Wachstein, M, and Zak, F G Intracellular bile canaliculi in the rabbit liver *Proc Soc Exper Biol & Med* 72, *234 (1949)*

18 Knisely, M H Microscopic observations of the circulatory conditions in the living frog liver lobules *Anat Rec* 73, *(Suppl 2) 69 (1939)*

19 Knisely M H, Bloch, E H, and Warner, L *A preliminary account of the structure and mechanical functioning of living frog liver lobules, and of microscopic observations of the selective phagocytic removal of coated particles from flowing blood by the sinusoid lining von Kupffer cells Liver Injury* Hoffbauer, F W, Editor Trans Fourth Conf New York, Josiah Macy, Jr Foundation, 1945 (p 21)

20 ——— *Selective phagocytosis* I Microscopic observations concerning the regulation of the blood flow through the liver and other organs and the mechanism and rate of phagocytic removal of particles from the blood *Det klg Danske videnskab selskab Biol Skrift* IV, *N 7 (1948)*

21 Loeffler, L., and Nordmann, M Leberstudien I Teil Die Leber bei der Verdauung von Normalkost, nach Fett, Glykogen und Eiweissfutterung im Hungerzustande und unter der Ein

wirkung von Adrenalin, Chloroform, Phosphor, Phlorhizin und Insulin Nach mikroskopischen Untersuchungen der Leber lebender Saugetiere *Virchows Arch Path Anat* 257, *119 (1925)*
22 WAKIM, K G, and MANN, F C The intrahepatic circulation of blood *Anat Rec* 82, *233 (1942)*
23 ELLINGER, P, and HIRT, A Eine Methode zur Beobachtung lebender Organe mit starksten Vergrösserungen im Lumineszenzlicht (Intravitalmikroskopie) *Handb d biol Arbeitsmeth* 5, *2/2), 1753 (1930)*
24 PICHOTKA, J Tierexperimentelle Untersuchungen zur pathologischen Histologie des akuten Hohentodes *Beitr path Anat* 107, *117 (1942)*
25 MÜLLER, E, and ROTTER, W Über histologische Veranderungen beim akuten Hohentod *Beitr path Anat* 107, *156 (1942)*
26 KRITZLER, R A Acute high altitude anoxia, gross and histologic observations in twenty seven cases *War Med* 6, *369 (1944)*
27 TROWELL, O A The experimental production of watery vacuolation of the liver *J Physiol* 105, *268 (1946)*
28 LEWIS, R B, and HAYMAKER, W High altitude hypoxia observations at autopsy in seventy five cases and an analysis of the causes of the hypoxia *J Aviation Med* 19, *306 (1948)*
29 GILLMAN, J, and GILLMAN, T Anoxia and the liver with special reference to shock and chronic malnutrition *South African J M Sc* 13, *11 (1948)*
30 GRAFFLIN, A L, and BAGLEY, E H Studies of hepatic structure and function by fluorescence microscopy *Bull Johns Hopkins Hosp* 90, *395 (1952)*

HEPATIC HISTOCHEMISTRY

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THIS WILL BE an introduction to a discussion on hepatic histo chemistry but I shall try to review some parts of the work done in our laboratory in Lund on cytochemistry of the liver with respect to the nucleic acid and protein metabolism

Recently there have been used two technically different ways of approach for investigations within this field The first is the study of sections from livers with the aid of optical methods and the other is the study of particles isolated from the liver cell cyto plasm and then investigated by physicochemical and chemical means The greater part of our work in Lund has been done with optical methods but in some recent experiments we have considered the possibility of correlating the findings thus obtained with those obtainable through analysis of particulate isolated fractions

It has long been known that starvation is associated with pro found changes in the cytologic appearance of the liver cell cyto plasm One of the most prominent of these changes is the dis appearance of basophilic cytoplasm inclusions These inclusions can be made conspicuous by several methods using basic dyes for instance the classical method with the pyronine staining

Figure 79 shows the picture obtained in the normal liver cell by a staining reaction the nucleoli within the nuclei can be seen and in the cytoplasm the basophilic inclusions Berg (1) made a com prehensive investigation in 1932 of the liver from several European and South American species and found the same morphologic features everywhere The same picture is also obtainable in man For experimental purposes the livers of Amblystoma rat mouse rabbit and dog have been used a good deal

To make an analysis of any process by cytological or cytochemical methods using a staining procedure certain things must be known about the method used The first question to be answered is about the specificity i e that under specified conditions the stainability of certain structures is due to the presence of the substance to be investigated forming part of those structures Secondly the method

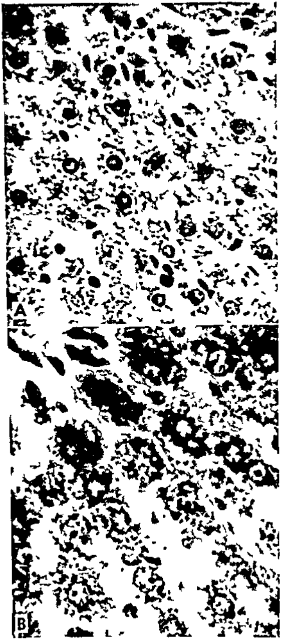


Fig. [illegible]
A [illegible])
B [illegible] sect on in [illegible]ltraviolet [illegible]

must be of the progressive type, reaching an end point, beyond which no more dye will be bound to the section following prolonged stay in the staining solution The fulfillment of the second demand is a prerequisite for getting reproducible results

We have found the gallocyanin chrome alum method (2,3,4 5) to be the ideal one for our purposes If the sections are left in that solution for twelve hours or for three weeks there is the same picture, prolonged staining will not change the pictures obtained, neither qualitatively nor quantitatively

For testing the specificity of the gallocyanin chrome alum staining different methods can be used One of these is the treatment of the sections with crystalline ribonuclease, which can be shown free of proteolytic activity As seen in Figure 80, after digestion

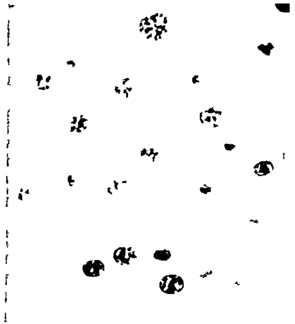


FIGURE 80 Section fr ver 7 microns on. Digesti 0 1 mg crystalline ribon 100 ml. McIlva pH 7 0 for at 37° Gallocyanin-chr ining at pH

the stainability is the nucle wher basophilic cytopla and the e are no longer stain t indic the cytoplasm and h d of ribonucleic acid tru

stainable chromatin, including the well marked accumulation around the nucleoli, should contain desoxyribonucleic acid With the aid of the Feulgen reaction, it is possible to show that this is the case, as visualized in Figure 81A Thus, these two staining methods indi-

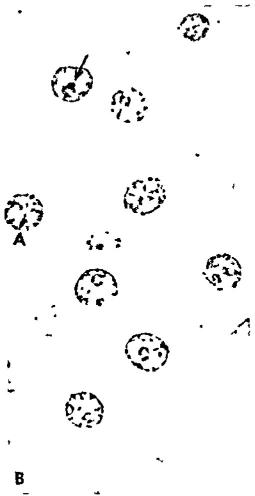


FIGURE 81 Feulgen reaction 5 microns Carnoy fixation

A The arrow indicates the Feulgen positive material forming the nucleolo-associated chromatin. (x1000)

B Section from animal starved for 5 days The absence of nucleolo-associated chromatin is conspicuous

cate that in the liver cell, the ribonucleic proteins are localized to the cytoplasmic inclusion and form the main bulk of the nucleoli, the chromatin net is composed of desoxyribonucleic acids which accumulate around the nucleoli, forming the 'nucleolo associated chromatin' (6)

For localization of nucleic acids within sections, other means can also be used We have investigated the distribution of ultra violet-absorbing substance at 257 mμ in the unstained sections from liver and determined the distribution of ash remnants after micro incineration

The ultraviolet microscope used is the classical Kohler design with a spark between rotating electrodes Figure 79B gives the picture of normal liver cells from an unstained section of frozen dried material photographed at 257 mμ The strongly absorbing structures consist of the nucleus with the chromatic net, the nucleolo associated chromatin, and the nucleolus, in the cytoplasm there are inclusions which can be proved to correspond to the basophilic bodies in the gallocyanin chrome alum stained preparations (3,4)

For this investigation, frozen dried and Carnoy-fixed sections from liver were treated with chloroform and glycerine, and studied mounted in glycerine between quartz glasses The absorption at 257 mμ shown can be looked upon as caused by purines, pyrimidines incorporated in the nucleic acids and by some cyclic amino acids forming part of proteins The absorption of these amino acids, tyrosine, trytophane, and phenylalanine is considerably weaker than that of the nucleic acid molecule in corresponding concentrations Nevertheless, as judged from photomicrographs obtained at 257 mμ the absorption shown by different structures might be caused by a high concentration of proteins containing those amino acids or by a low concentration of nucleic acids

With the Kohler microscope, it is possible to register the absorption of a small part of a cell in different wave lengths and thus to obtain the spectral distribution of the light absorbed within the ultraviolet region (7,8) Plotting the curves from such measurements shows the presence or absence of the characteristic absorption curve for the nucleic acid molecule in the absorption picture of a certain structure Thus, as shown in Figure 82A, absorption measurements from points within the nucleoli and the cytoplasmic inclusions give the characteristic absorption maxima at 257 mμ, while measuring points within the ground cytoplasm in between the inclusions fail to do so These findings support the results reached

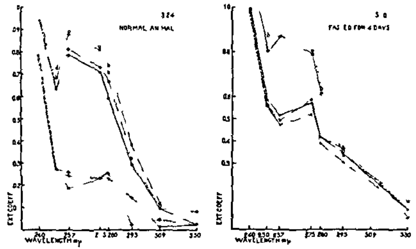


FIGURE 82 Ultraviolet absorption spectra from rat liver cells Lower group of curves from measuring points in inclusion free cytoplasm ("ground cytoplasm) Upper group of curves the two lower curves in Figure 82A from cytoplasm inclusions the two upper curves in Figure 82A and the curves in Figure 82B from nucleoli
A Normal animal on protein-containing diet
B Animal after 4 days' starvation
Reprinted by permission from Lagerstedt S Effect of microincineration on the proteinaceous inclusions in normal rat liver cytoplasm *Acta anat* 3 190 (1947)

with the gallocyanin chrome alum method in connection with the ribonuclease test

In our work we have not used the ultraviolet absorption method for quantitative but only for qualitative purposes to show the presence or absence of nucleic acids in certain structures

As mentioned we have tried to confirm these results by using the microincineration test As the nucleic acid molecule contains approximately 25 per cent phosphoric acid such structures which contain nucleic acids are predestined to leave a considerable ash residue as compared to most other structures It is also possible to show that the ultraviolet absorbing basophilic inclusions in the liver cell cytoplasm give rise to distinct ash residues when the microincinerated sections are investigated under dark field illumination (9 10 11 12 13 14) (Figure 83A) Of course an ash residue in the incinerated section tells almost nothing about the qualitative or quantitative composition of cell structures but as a confirmation of the results reached with other methods it is a valuable tool

Using these different methods we have investigated the liver of adult white male rats submitted to starvation for different lengths

cate that in the liver cell *the ribonucleic proteins are localized to the* cytoplasmic inclusion and form the main bulk of the nucleoli, the chromatin net is composed of desoxyribonucleic acids which accumulate around the nucleoli, forming the 'nucleolo associated chromatin" (6)

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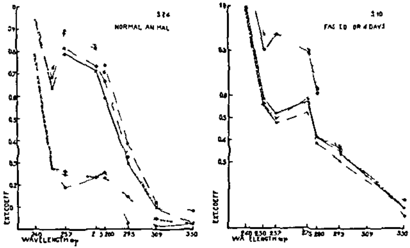


FIGURE 82 Ultra ol t absorpt on spectra fro n rat l ver cells Lo er group of

Reprinted by perm ss on from Lagerstedt S Effect of micro ncinerat on on the prote nic o s inclus ons in normal r t l er cytopl sm *Acta anat* 3 *190 (1947)*

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Using these different methods we have investigated the liver of adult white male rats submitted to st rvation for different lengths

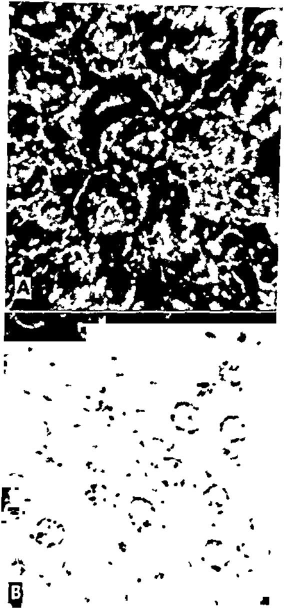


FIGURE 83 Microincinerated sections 4 microns Carnoy fixation Dark field (x100)
A Normal picture
B Section from animal starved for 5 days



FIGURE 84 Sections from animals starved for 5 days 7 microns Carnoy fixation. 1000)

of time and afterwards re fed with diets containing differing amounts of protein The normal animals were fed a diet containing 20 per cent casein as protein source and an adequate amount of salt and vitamins the starved animals got nothing but water for 5 days There are some striking changes observable by comparing sections from the well nourished animals liver with those from the liver of the starved animals In Figures 84A and B the basophilic cyto plasm inclusions have disappeared as have the ultraviolet absorb ing ones and there is a considerable reduction of ash residue in the starved animal (Figure 83B) Within the nuclei the nucleolo associated chromatin has been reduced to only slightly stained rims around the nucleoli as seen in Figure 81B

Shorr What is the slightly stained rim?

Lagerstedt Desoxyribonucleic acid

In making absorption measurements in the cytoplasm from these starved animals the curves obtained are all of the ground cyto plasm type with no selective absorption at 257 mμ (Figure 82B)

Watson What kind of densitometer do you use when measuring the curves of the nucleolus?

Lagerstedt All the curves shown here are obtained through density measurements on photographic plates

Watson Is the field cut down so there is nothing but the nucleolus?

Lagerstedt Yes or any other point of interest on the 9 x 12 cm photographic plate used The plates themselves are calibrated with the aid of a rotating step sector projected during the exposure on a piece of the plate which is free from tissue in the field of view From several plates obtained using light of different wave lengths it is possible to measure the absorption of any desired point in the tissue and so plot the absorption curves as shown on the graphs

Hartroft What is the section thickness?

Lagerstedt Five microns

Hartroft How did you control it?

Lagerstedt I did not because I had no particular reason to for these investigations Of course with a nucleic-acid containing struc ture in the cytoplasm with a depth of 3 microns and a section thick ness of 5 microns ground cytoplasm will be under or above or on both sides of that structure This fact of course will affect the outcome of a quantitative measurement In some cases it is possible to avoid that effect as for instance in an investigation made some years ago (8) where it was possible to accumulate the absorbing substance in one layer in the cells with the aid of ultracentrifugation In the investigations reported here the main purpose was to obtain

qualitative information as to the composition of the cytoplasm which was fairly well done through studies of curves from measuring point through absorbing structures in the cytoplasm and between them ("ground cytoplasm) Thus it was possible to determine if the concentration of nucleic acids was so high as to give the characteristic curve or if this concentration was too low which is the qualitative information sought If in the first case there has been some contamination also from surrounding "ground cytoplasm " this is of quantitative but not of qualitative importance

Hartroft Is it possible to compare fields within the same section?

Lagerstedt Yes

Schwartz There is a method of photographing cells in such a way as to reveal those areas in which nucleic acid is concentrated distinctly from those areas in which protein is most concentrated As Dr Lagerstedt pointed out nucleic acids are photographed with 257 mμ light but proteins also contribute to the absorption at this wave length Similarly photography at 280 mμ may reveal a picture due predominantly to protein but here again nucleic acids also contribute to the absorption pattern As Dr Lagerstedt has mentioned one can plot absorption spectra of these areas to determine relative concentrations of protein and nucleic acid

I should like to suggest a simple trick which enables one to visualize relative concentrations of protein and nucleic acid in the same cell First one photographs the field with 257 mμ light and develops this film as a positive secondly one photographs the same area with 280 mμ light and develops this film as a negative These are then exactly superimposed and the combination of the two photographed In this way not two shades of darkening (background and absorbing areas) are obtained but rather three shades (1) a background shade (2) a shade darker than background due to relatively high concentrations of nucleic acid and (3) a shade lighter than background due to relatively high concentration of protein (This technique has been successfully used in unpublished studies of bone marrow cells)

Pollister There is a more direct way than that which we prefer to use Dr Lagerstedt has outlined the basis for it in speaking of how nuclease is used to establish the specificity of gallocyanin An estimation of the part of the ultraviolet light loss which *is due to* protein can be made by taking two ultraviolet photographs or making two photometric measurements of the same material one before the other after removal of the nucleic acid by nuclease digestion or trichloracetic acid extraction the difference between

the densities of these two is entirely due to nucleic acid This is precisely comparable to the blank which a biochemist uses to cancel out all sorts of nonspecific light loss in a photometric analysis A biochemist would never work without such a blank

Lagerstedt It was known very early primarily through the work of Berg that the basophilia of liver cell cytoplasm has a close connection with the amount of protein given in the diet He was able to show that the reappearance of the basophilic bodies only followed the administration of protein diets to the starved animal

It has been shown in repeated starvation and refeeding experiments that starvation is followed by a decrease and that refeeding of a protein diet is followed by an increase in the amount of ribonucleic acid in the liver Kossel as early as 1882 noticed this fact It has been thoroughly studied in recent times by Davidson and Waymouth by Kosterlitz and by several others

Through the different cytological and cytochemical methods previously discussed it has been possible to study in detail the changes occurring within the liver cells associated with starvation and refeeding experiments

As pointed out in several works by Caspersson and his associates increase in the nucleolar mass is highly characteristic for cells in an active stage of protein metabolism To be able to follow *these changes quantitatively in the liver we have determined the total* amount of nucleolar material using the so called Chalkley method As we think it justified to lay great stress upon the results reached by that method I should like to make an outline of the principles adopted

The Chalkley method is based on the random distribution of some fixed points situated in the plane of the ocular diaphragm among the structures visible in the microscopic field of view After chance focusing on a number of visual fields it is possible to determine how often the image of the end points of four straight lines coincide with the pictures of certain structures in the sections Such a contact is designated as a *hit For further explanation see* Figure 85

After focusing in this manner a good many times the ratio can be calculated between the number of hits on one type of structure (for instance nuclei) and the number of hits on another type (for instance *nucleoli or cytoplasm*) For statistical treatment of the material the observations are divided into groups with regards to one of the structures Thus 25 hits on nuclei were taken as a unit and the number of hits on other elements had reference thereto

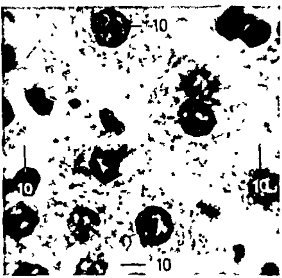


FIGURE 65 Photo of visual field illustrating the technique for measuring the ratio of hits on nucleoli to hits on nuclei and hits on cytoplasms to hits on nuclei In the visual field, four straight lines are seen with "10" at one end The other end is used as "contact point" The contacts between these ends and the clearly visible picture of nucleolus or cytoplasm are recorded as "hits In this case, then the reading will be 1 hit on nucleolus (above) which is also recorded as 1 hit on nucleus 2 hits on cytoplasm (in middle) The end point of the line at the bottom is in contact with the space of a capillary and therefore recorded as 0

In this way, it is possible to calculate the ratio of hits on nucleoli to hits on nuclei, for example, 225 hits on nuclei correspond to 40 hits on nucleoli, giving a ratio of 0 18 with a standard deviation s = 0 06 It is, of course, necessary to determine how many hits on nuclei have to be registered in order to give a stable mean value

In recording the number of hits on different elements at different levels in the section through focusing up and down, the ratios express the fraction of unit volume occupied by one structure to the fraction of unit volume occupied by another

Hartroft When dealing with such small structures as nuclei, are not errors introduced by the phenomenon of parallax? If you move your eye a little to the right or left does not the point shift in relation to the structures in the slide?

Lagerstedt No You have the points in a central position in the field of view With respect to the microscope used, certain demands must be fulfilled The serviceability of the method is chiefly limited by two factors (*1*) the resolving power of the objective employed

and (2) the sharpness and dimensions of the registering points employed Provided that the given structures stain distinctly, we may set the lower limit for the permissible size of the objects at about 0 2 microns The registering points ought to be of such dimensions that the area in the microscope picture covered by them is equal to the dimensions of the smallest, distinct, observable structures, i e, 0 2 microns

We do not focus up and down As I said, in focusing up and down you get the fractions of unit volumes, if you record hits on structures situated above or below each other It has proved very difficult in our material to determine the transition between different elements So instead of this, we have determined the number of hits on projection pictures of nuclei, nucleoli, and cytoplasm observable in a given visual field The procedure is evident from Figure 85

Using this method, together with the cytological and cytochemical ones, we made the following basic experiments Male rats were starved for five days, after having been fourteen days on a standard diet which contained 20 per cent casein as protein source During those five days they were given just water Among approximately 300 starved rats, only three died in consequence of starvation These starved rats were divided into two groups Those in the first one received a diet containing 20 per cent casein, which I shall refer to as 'the high protein diet," and the other received a low protein diet' with brewer's yeast as the source of protein giving 0 3 per cent total nitrogen in analysis of that diet

Table XVII shows the effect of fasting on the total nitrogen content of the liver, counted as nitrogen in milligrams per 100 Gm initial body weight In the normal rats used by us it was found that the body weights decreased from 175 ± 4 Gm to 127 ± 5 Gm after five days of starvation In both cases, standard deviations were about 19 As shown in Table XVII, there was a decrease from 153 to 94 milligrams nitrogen per 100 Gm initial body weight during this period

Looking at the pictures obtained from livers during this time of starvation it becomes obvious that the cytoplasmic ribonucleic acid containing inclusions disappear first from those cells which are situated in the periphery of the lobules After twenty four hours' starvation cells situated centrally contain the same amount of cytoplasmic inclusions as normal liver cells whereas the cells in the periphery show the appearance of starved cells, lacking cytoplasmic basophilic inclusions

TABLE XVII

Effect of Fasting on the Total Nitrogen Content of the Liver

Days of Fasting	No of Rats	Mean Initial Body Weight	Mg N/100 Gm Initial Body Weight
0	18	175 ± 4 s = 18	153 ± 3 s = 14
1	7	170 ± 8 s = 21	141 ± 3 s = 9
2	8	162 ± 12 s = 35	132 ± 6 s = 17
3	7	168 ± 8 s = 22	120 ± 5 s = 13
4	7	169 ± 5 s = 13	103 ± 5 s = 14
5	44	172 ± 3 s = 22	94 ± 2 s = 14

There are, nevertheless, some differences in these centrally situated cells as compared to cells from normal animals In the normal cell, it is common to notice basophilic substance in close connection with the nuclei in fact indistinguishable from the nuclear membrane This localization of the inclusions is a most uncommon one after starvation, even in cells where basophilic bodies are still detectable This observation seems to us of a certain importance for the understanding of the mechanism underlying the disappearance and reappearance of the ribonucleic proteins in the cytoplasm

In the liver, there is the advantage of being able to study many cells in the same stage of metabolic activity If one is interested in following the sequence and character of the cytological and cytochemical events accompanying the reappearance of the nuclear and cytoplasmic nucleoproteins all these cells ought to be at the same stage i e be indistinguishable from each other by the methods used at the start of the experiments and this demand is fulfilled in the liver only after five days starvation, earlier, there are always some basophilic inclusions remaining in the most centrally situated cells So to perform the study under clear cut experimental conditions we have used animals starved for five days before refeeding

As will be more evident from the following we think that the retraction of basophilia from the vicinity of the nuclei early in starvation is a sign of decreased activity in the protein metabolism

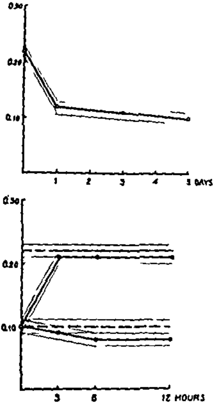


FIGURE 86 [illegible] hits on nucleoli to hits on nuclei [illegible] tion to da[illegible] nucleolar [illegible] starvation (●) Corresponding deter[illegible] diet (◑) ○ = value obtained after 5 days starvation The thin lines represent the standard errors for the means Ordinate Mean of ratios of hits on nucleoli to hits on nuclei For the corresponding figures see Table XVIII

There are other signs, however, which point in the same direction Among them are the results of the measurements of the nucleolar mass

As shown in Figure 86 and from the data in Table XVIII, it is obvious that even on the first day of starvation the nucleolar mass had decreased to half the amount of that in the animal fed normally After that initial decrease, no further statistically verifiable changes were observable during the length of time involved in the experiment

Stetten If I understand this correctly, this is based on the ratio of hits

Lagerstedt Yes, it is

TABLE XVIII

Changes in the Ratio of Hits on Nucleoli to Hits on Nuclei* due to (*1*) Inanition, (*2*) Refeeding the High Protein Diet, (*3*) *Refeeding the Low Protein Diet

INANITION

Inanition Period in Days	Ratio Hits on Nucleoli to Hits on Nuclei	
0	0.17 ± 0.01	s = 0.10
	0.21 ± 0.03	s = 0.09
	0.22 ± 0.03	s = 0.10
	0.25 ± 0.03	s = 0.10
	0.24 ± 0.03	s = 0.09
1	0.12 ± 0.02	s = 0.06
	0.12 ± 0.01	s = 0.03
	0.11 ± 0.02	s = 0.05
	0.11 ± 0.01	s = 0.05
3	0.13 ± 0.02	s = 0.08
	0.13 ± 0.02	s = 0.06
	0.09 ± 0.01	s = 0.05
	0.10 ± 0.02	s = 0.07
5	0.07 ± 0.02	s = 0.05
	0.12 ± 0.02	s = 0.06
	0.08 ± 0.01	s = 0.03
	0.13 ± 0.02	s = 0.06
	0.09 ± 0.01	s = 0.05

REFEEDING AFTER 5 DAYS STARVATION

Feeding Time in Hours	Ratio Hits on Nucleoli to Hits on Nuclei: High Protein Diet		Low Protein Diet	
3	0.23 ± 0.02	s = 0.06	0.08 ± 0.01	s = 0.04
	0.18 ± 0.02	s = 0.08	0.10 ± 0.02	s = 0.06
	0.22 ± 0.02	s = 0.07	0.08 ± 0.01	s = 0.03
6	0.19 ± 0.01	s = 0.04	0.09 ± 0.01	s = 0.04
	0.21 ± 0.02	s = 0.06	0.08 ± 0.02	s = 0.07
	0.22 ± 0.02	s = 0.08	0.07 ± 0.02	s = 0.06
12	0.20 ± 0.02	s = 0.05	0.07 ± 0.95	s = 0.05
	0.21 ± 0.02	s = 0.07	0.08 ± 0.02	s = 0.05
	0.22 ± 0.01	s = 0.05	0.09 ± 0.02	s = 0.05
24	0.22 ± 0.02	s = 0.08		
	0.21 ± 0.01	s = 0.05		
36			0.15 ± 0.01	s = 0.04
			0.13 ± 0.01	s = 0.03
			0.17 ± 0.02	s = 0.08

Stetten Isn t the ratio of hits related to area of cross section rather than mass or volume? Isn t the mass or volume related perhaps to the 3/2 power of the ratio?

Lagerstedt The figures from the measurements which I have shown in Figure 86 are the ratio of the hits on surfaces as explained previously I am sorry if I have inferred that volume was considered for I meant surfaces

Stetten These values presumably could be translated into mass by raising them to the 3/2 power?

Lagerstedt Yes with certain precautions taken I should like to say something about that in just a moment

For an appraisal of the measurements two factors have to be considered in particular (*1*) variations in the size of the nuclei and (2) variations in the number of nucleoli per nucleus As the figures here shown refer to the surface of nuclei as a unit it is obvious that an increase in the individual diameter of the nuclei may give the same result as a decrease in that of the nucleoli Measurements of 300 nuclei from five different animals normal and starved for five days gave a decrease in the nuclear diameters from 7 2 to 6 8 μ approximately This decrease in size of the standard makes the decrease in ratio even more significant

For the other problem it is of importance whether the total measured nucleolar surface is derived from single nucleoli the individual diameter of which is subject to variation For if two smaller surfaces give a total surface of the same size as that of one larger one then the two smaller surfaces together will represent a smaller volume than the big one assuming that they all are spherical in form It is obvious therefore that if in the specimens measured the nucleolar surface in the starved animals is distributed on fewer single nucleoli than in the normal animal a diminution of the surface will not correspond to a proportional decrease in volume In a border case therefore their smaller total surface may be due to divisions of the nucleoli without any loss in volume Therefore the number of nucleoli per nucleus was determined and it was found that the starved animals nuclei showed a greater number of nucleoli per nucleus than in the normal animal therefore the diminution observed in the surface of the nucleolar mass must correspond to an even greater difference in volume

We started the refeeding experiments with these starved animals giving them the high protein diet and the low protein diet

Shorr The vitamin content is always the same?

Lagerstedt Yes it is

Smetana Is there any difference in the distribution of the cytoplasmic inclusions at different times of the day due to feeding or special function?

Lagerstedt I have tried to figure that out it should perhaps be possible to do so with the Chalkley method but I have not used this method for that purpose as yet Just in looking at the sections there are no such changes as those of glycogen bile acids as shown by Forsgren

In giving the animals the high protein diet the aim is of course to study the mechanism involved in the formation of ribonucleic acids and proteins This formation as is known from chemical data will take place after some time But there is one big difference between studies with cytochemical and biochemical methods there may be very distinct changes within cells restricted to definite areas and/or to parts of the single cell before any will be found macrochemically in the over all values for nucleic acids or proteins In our experiment with the high protein diet this fact came out most noticeably The first changes observable by cytological methods following the administration of the diet occurred within three hours after the feeding of the animals The first demonstrable change occurred in the nucleolar apparatus This is obvious from the graph in Figure 86 and from Table XXIII

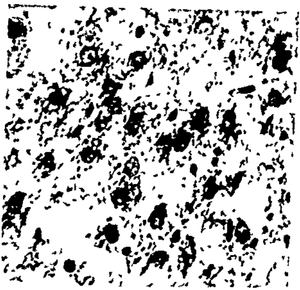
As seen from Figure 86 the amount of nucleolar material increases 100 per cent within the first three hours after this no statistically significant change occurs with this increase the ratio is the same as in the normal animal

Giving the starved animals the low protein diet has no statistically significant effect on the ratio (lower curves in Figure 86) within the twelve hours studied As seen from Table XXIII there is noticeable a statistically significant increase in the ratio values after 36 hours This increase which we have not analyzed further as yet is of special interest in connection with the report by Stowell (15 16) who found large nucleoli after about three months on a low protein diet but rather low amounts of ribonucleic acids in the cytoplasm

Studies of the pictures obtained with the gallocyanin-chrome alum method the Feulgen reaction the ultraviolet microscope and microincinerated sections give the following results Preparations from animals after three hours on the high protein diet showed only changes within the nuclei as revealed by the Feulgen method The starved animal had very little desoxyribonucleic proteins surrounding the nucleolus but after three hours an accumulation of that

substance is noticeable, forming the nucleo associated chromatin (Figure 81A) The cytoplasm has the same appearance as in the starved animal

After six hours, there is a most characteristic picture all the nuclei in the cells around the central vein are surrounded by heavily staining substance (Figure 87) which is also clearly seen in the



F... 87 ... 7 from animal starved ...rons Carnoy fixation (×400) Cf Figures

ultraviolet microscope and yields a distinct ash residue upon micro incineration It is possible that this accumulation is the result of incorporation of glycogen in the cytoplasm which can be shown with the Best method We studied that problem in particular, and we found that it is always possible to show unchanged 'ground cyto plasm" outside the concentrations around the nuclei There are also other conditions (regeneration following bleeding or partial hepatec tomy etc) where no pronounced incorporation of glycogen in the cytoplasm occurs but where nevertheless the basophilia is concen- trated in the area around the nuclei in close contact with the nuclear membrane

Watson That is ribonucleic acid?

Lagerstedt Yes The curves obtained from the ultraviolet meas urements show also the selective absorption at 257 millimicrons I

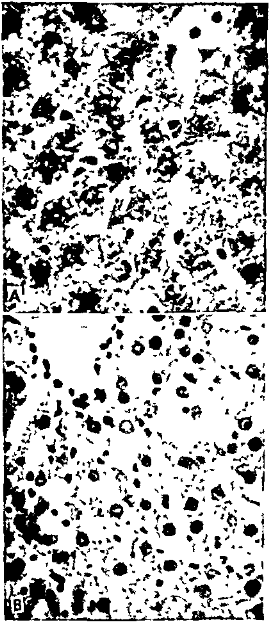


FIGURE 68. Gallocyanin chrome alum, pH 2.3. Carnoy fixation. 7 microns.
A. Section from animal starved for 5 days and then given the high protein diet for 24 hours.
B. Starved animal given the low protein diet for 24 hours.

emphasize that these curves are not for comparative quantitative purposes but only for demonstration of the chemical composition of the substance

After twenty-four hours on the high protein diet, the pictures obtained are very similar to those from the normal animal (Figure 88A) *It is quite clear that the mobilization of the ribonucleic proteins* develops first in the centrally situated cells and later on in the cells in the periphery of the lobules

Comparing these findings with those from the animals which have received the low protein diet for the corresponding length *of time (Figure 88B), it is obvious that there is no accumulation* of ribonucleic acid visible

Hartroft And this followed five days' starvation?

Lagerstedt Yes, starved for five days and given a low protein diet

The basophilia seen in these sections depends not upon the presence of ribonucleic acid, as it can't be removed by ribonuclease treatment

I think it would be of some value to make a statement with respect to the way we used the gallocyanin-chrome alum method on these preparations With the gallocyanin chrome alum method, it is possible to get the picture of the nucleoprotein containing structures at pH 1 7 in the staining solutions If the staining is performed at pH 2 7, there is a co-staining of structures which do not contain nucleoproteins That means that after treatment with ribonuclease the structures are still stainable with the staining solution at that pH Figure 89 and [illegible] the amounts of total nitrogen [illegible] is a very early increase in [illegible] increase which is statistically significant

In the scheme in Figure 90, I have tried to visualize the principal changes in *the liver cell during starvation followed by feeding* with the high protein diet I also want to give an account of the sequence of events involved in the processes of regeneration The timing is of particular interest It shows that the first noticeable change is the increase of the nucleolar mass by about 100 per cent after three hours *on the diet* while there are still no changes observable in the cytoplasm After about six hours on the diet, the first ribonucleic proteins are observable in the cytoplasm and at that time in close connection with the nuclear membrane This stage corresponds to the first statistically significant increase in the total

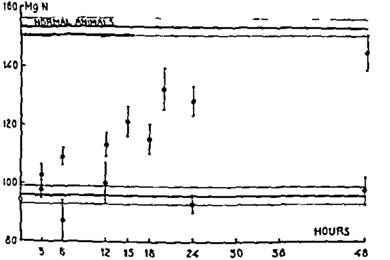


FIGURE 89 Graph of changes in the total nitrogen content of the liver (mg. N per 100 Gm initial body weight) in animals given the high protein diet (●) or the low protein diet (◐) after 5 days starvation (○) The vertical lines attached to each value represent the standard error The horizontal lines represent the mean value and the standard error for this mean above for normal animals below for the experimental animals on low protein diet here recorded Cf Table XIX

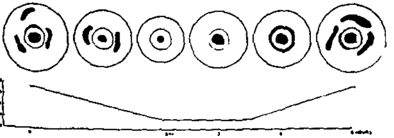


Figure 90 [illegible]

nitrogen of the livers After forty eight hours the normal picture is restored

The difference between these animals and the starved animals on the low protein diet is primarily localized in the cytoplasm As described above it is possible to observe an increase in the amount of nucleolar material present after fifteen hours on the low protein

TABLE XIX

Effect on Total Nitrogen Content of the Liver in Animals Starved for 5 Days and Then Given the High Protein Diet or the Low Protein Diet

Hours on Diets	HIGH PROTEIN DIET			LOW PROTEIN DIET		
	No of Rats	Mean Initial Body Weight	Mg N/100 Gm. Initial Body Weight	No of Rats	Mean Initial Body Weight	Mg N/100 Gm Initial Body Weight
3	20	171 ± 5 s = 21	98 ± 3 s = 12	6	163 ± 7 s = 16	103 ± 4 s = 10
6	31	177 ± 3 s = 19	109 ± 3 s = 18	7	189 ± 10 s = 26	87 ± 7 s = 17
12	14	178 ± 7 s = 25	113 ± 4 s = 13	9	171 ± 6 s = 17	100 ± 7 s = 20
15	5	164 ± 11 s = 25	121 ± 5 s = 12			
18	9	157 ± 5 s = 15	115 ± 5 s = 16			
20	4	163 ± 4 s = 9	132 ± 7 s = 14			
24	17	204 ± 9 s = 38	127 ± 5 s = 22	9	167 ± 5 s = 15	93 ± 3 s = 10
48	16	188 ± 6 s = 23	145 ± 6 s = 26	18	182 ± 7 s = 20	98 ± 5 s = 15

diet and Stowell has shown that after longer periods on a low protein diet the nucleoli become enormous but there is little ribo nucleic acid in the cytoplasm

One of the main points according to the general scheme of Caspersson and his co workers is that there should be an accumulation of proteins and nucleoproteins around the nuclei this substance should be synthesized there under the influence of the nucleus The first sign of those substances is around the nucleus but I don t think we have enough information yet to know whether this is accumulating from the cytoplasm toward the nuclear membrane or from the nucleus to the cytoplasm There is one point I think I have been able to show namely that changes within the nucleolus precede those in the cytoplasm That finding is not only from the experiment reported here We have several others For instance animals bled (2 or 3 grams of blood from a 100 gram rat) give a picture like that of five days starvation

Shorr How soon?

Lagerstedt After four hours and after seven or eight hours regeneration of the cytoplasmic ribonucleic proteins is found with an increase in the nucleolar mass and accumulation around the nucleus in the cytoplasm with the usual sequence of events

In the investigations by Stowell there is a large nucleolar mass but only small amounts of ribonucleic material in the cytoplasm on the other hand there is so far as I know no evidence of the presence of a small nucleolar mass together with accumulation of nucleoproteins around the nuclei

Stetten In the partially hepatectomized but well nourished rat I would think each proliferating cell of the liver is producing more nuclear material I wonder if you have investigated this aspect

Lagerstedt Stowell has and he showed the same general scheme as that pointed out here He also studied tumor formation I have forgotten what carcinogenic substance he used He found early changes which led to hepatoma and he showed the accumulation of ribonucleic proteins around the nuclei Recently some other work has been done by Stenram among others studying the effect of vitamin B_{1} on these protein forming systems some evidence is accumulating that in vitamin B deficient animals the protein forming mechanism is impaired

Before going on I wonder whether Dr Pollister will add his comments to the discussion at this point

Pollister This information which Dr Lagerstedt has presented is really the only acceptable evidence that the nucleolus is closely

correlated with protein synthesis This might seem a surprising statement since the Caspersson school has repeatedly stressed this relationship But their approach has never been as broad as this nor has the matter been approached with a statistically significant number of animals It is one thing to pick out a few cells which have large nucleoli or to point out that where there is growing tissue there are large nucleoli it is an altogether different thing to nail the matter down as Dr Lagerstedt has done by setting up an experiment with a large number of animals and showing statistically that there is a definite quantitative increase in the amount of nucleolar material when the protein nitrogen increases Unless we come upon an exception and none has turned up so far here is at least one cytological finding on which we can build namely that there are marked changes inside the nucleus in the way of nucleolar volume and configuration of chromatin which accompany protein synthesis

Recently in our laboratory Flax finished a quantitative study which relates the loss of the nucleolus at the end of oocyte growth and its subsequent reappearance in the early mouse embryo to changes in amount of protein and ribonucleic acid in the cell In brief these photometric measurements show that in the oocyte both protein and RNA increase steadily so long as the nucleolus is present then drop soon after it is lost The nucleolus reappears in the embryo at the four cell stage of the blastomere nuclei the RNA and protein begin to increase distinctly later at the six to eight cell stage

Best Are there any comments?

Waterlow Has anybody made any study of the ribonucleic acid in the choline deficient state?

Lagerstedt I dont know

Waterlow There are striking changes a large increase in nucleoli these appear in ten days or two weeks

Hartroft After fat has been rapidly mobilized from livers of choline deficient rats by restoring lipotropic factors to their diet the cytoplasm of the previously fatty hepatic cells lacks for a short period the normal basophilic staining reaction We have not however studied nucleic acids in such preparations by any specific histochemical procedure

Popper Two years ago I presented at the Liver Injury Conference (17) data pertaining to this questio owed a Figure which indicated that in ac fatty liver a by ethionine the basophilia as indi ronine is reduced with

out significant alterations of the total nucleic acid content as referred to initial body weight I refer to chemical determinations The same holds true as Farber, Koch Weser and Szanto (18) showed in our laboratory for the pentose nucleic acids, and the desoxypentose nucleic acids also did not alter

Lagerstedt In all these types of experiments it is very hard to say something definite about a single case I have the general feeling one must put the case in the sequence of experiments to evaluate the different changes It would be interesting to perform an experiment on those choline rats and see the changes in nuclear volume and nucleolar volume and try to get something out of that We have had no experience at all with that type of animal

Pollister Not only does it appear that there is desoxyribose nucleic acid which is not interconvertible with the ribose type but it appears also that we cannot adopt an obvious scheme and say that the nucleic acid in the cytoplasm necessarily came from the nucleus which is one conclusion which might be drawn from the sequence of events which Dr Lagerstedt has discovered Against this is evidence that in base content and in relative rates of incorporation of P^{32} there are differences between nuclear and cytoplasmic RNA

Lagerstedt I think of the general problem in the following way *It is possible to demonstrate ribonucleic acid within the nucleoli* and in the cytoplasm the sequence of events associated with the formation of the cytoplasmic ribonucleic acids has been repeatedly stressed here It is a basic fact that the living cell is dependent upon the presence of the nucleus for performing normal metabolic activities The observation that the nucleolus and the basophilic cytoplasmic inclusion bodies stain in the same way with many basic dyes led to the assumption that material of nuclear origin could be transferred into the cytoplasm This should in some cases explain the mechanism underlying the influence of the nucleus on the cytoplasm

Two years ago I received a paper from Germany saying it had been found that in rat livers sometimes the nucleolus could leave the nucleus and appear in the cytoplasm This is a rather old concept already held by Kremer in 1933 (19) In his pictures of the frog liver there were seen many nucleoli in the cytoplasm This turned out to be incorrect as it could be proved that the nucleoli were transported into the cytoplasm by the edge of the microtome knife

correlated with protein synthesis This might seem a surprising statement, since the Caspersson school has repeatedly stressed this relationship But their approach has never been as broad as this, nor has the matter been approached with a statistically significant number of animals It is one thing to pick out a few cells which have large nucleoli, or to point out that where there is growing tissue there are large nucleoli, it is an altogether different thing to nail the matter down, as Dr Lagerstedt has done, by setting up an experiment with a large number of animals and showing statistically that there is a definite quantitative increase in the amount of nucleolar material when the *protein nitrogen increases* Unless we come upon an exception, and none has turned up so far, here is at least one cytological finding on which we can build namely, that *there are marked changes inside the nucleus, in the way of nucleolar* volume and configuration of chromatin, which accompany protein synthesis

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Popper Two years ago I presented at the Liver Injury Conference (17) data pertaining to this question and showed a Figure which indicated that in acute fatty liver as produced by ethionine, the basophilia as indicated by pyronine, is markedly reduced with

discussion of the technical difficulties encountered in the method used

Dr Pollister has had extensive experience in such problems Especially should I like to draw attention to the question about those situations where it seems impossible to stain the cytoplasmic ribonucleic acid which by chemical analyses can be proved to be there I should like to pass this question on to Dr Pollister

Pollister This is a very important point If a positive basophilia is obtained which is removable by nuclease digestion or chemical extraction it is certainly justified to conclude that the stained areas contained nucleic acid (21) The difficulty arises if ultraviolet absorption is not being used in the interpretation of a negative basophilia The following appears to be the method of chemical staining the only type of basophilia which can be used for reliable localization and estimation of nucleic acid in cells In the native state nearly all of the acidic groups of the nucleic acid are bound to basic groups of proteins that is in the form of protein nucleates If dye is to combine with the nucleic acid it must either displace the protein or attach to groups from which fixation has already broken the protein away from the nucleate combination

We may take as a model the purified tobacco mosaic virus which is the best known native nucleoprotein It was found that all the basic dyes bound by the native nucleoprotein could be accounted for through free acid groups of the protein (22) But if the protein were denatured by acid there were immediately available for dye binding many more acid groups in the nucleic acid When tissues are acid fixed as for chemical staining apparently something of this sort is done to the cellular nucleoprotein so that free nucleic acid groups result which will combine with the basic dye Whether or not there are any free acidic groups except those of protein in the living cell is not known though it has been discussed vigorously (23)

A method of determining the proportion of dye bound to amount of nucleic acid present in tissues can be employed this involves photometric measurements of ultraviolet absorption and of the dye This has been done rather extensively in our laboratory by Flax* He found that the ratio of dye bound to nucleic acid present in tissue was pretty constant in quite a number of mammalian tissues though there are some conspicuous exceptions When animal is compared with plant tissue or mammalian with amphibian the ratio is found to be significantly different.

* Unpublished observations

I think that neither by the method mentioned here, the purely morphologic approach, nor by analyses of chemical substances as previously described, is it possible to elucidate the problem of the interaction between nucleus and cytoplasm in the liver cell. Only through an intimate coordination of different techniques should these problems be tackled. This seems to me especially striking when one considers certain recent findings in another investigation of the isolated particulate fractions from the liver cell cytoplasm. Among those, one fraction consists of the mitochondria and another, presumably not homogenous, of microsomes, submicroscopic particles. These microsomes contain the larger amounts of ribonucleic acid and might correspond to the basophilic inclusions within the stained cell. It has been possible to study in some detail many of the metabolic processes connected with the isolated particles. On the other hand, we know a good deal about the metabolism in the cell in which the ribonucleic proteins of the cytoplasm are involved. One example of my point of view is that a coordination of these entirely different methods for investigating the liver cell metabolism beyond doubt could furnish valuable information.

Hartroft: After three months or more of dietary choline deficiency in rats, a rather striking qualitative change occurs in many of their hepatic parenchymal cells. Stainable fat appears within many of the nuclei. I have not encountered this in other pathologic states, and the only account in the literature that I have been able to find is in Henke Lubarsch. Glycogen within the nucleus is more commonly encountered in a number of pathologic states, but stainable fat in this position is quite rare.

Grafflin: Some years ago I encountered intranuclear sudanophil fat droplets in the liver cells of the normal guinea pig (20).

Hartroft: We have never observed stainable intranuclear fat in the livers of our normal rats.

Best: Dr. Lagerstedt, will you continue with your presentation.

Lagerstedt: There is one question which is often asked and which is of considerable interest from a practical point of view. Is it possible to draw conclusions about the functional state of the liver from the routine histopathologic preparation? When there are possibilities for investigations with all the special equipment mentioned before, there are no peculiar difficulties, but in most laboratories, such investigations are performed by the use of different staining procedures. Thus, the answer to the question is mainly a

dehydrated at low temperature In that way changes in the localization caused by the penetration of the tissue by fixatives is avoided as are the chemical changes accompanying the fixation in fluids

The other method consisted of submitting pieces of fresh tissue to ultracentrifugation The theory is that if cell constituents can be moved within the cells in the centrifugal field with unchanged

sing different

also present

Best I don t think that is quite clear

Lagerstedt In a certain type of cell for instance in the liver cell inclusion bodies will be found in the cytoplasm Using different types of fixatives they will always be found This shows that they are not formed under the influence of any particular type of fixative Now in centrifuging pieces of tissue the formed bodies in the nucleus and cytoplasm arrange themselves under the influence of the gravitational forces The ability to be displaced under the influence of gravitational forces of certain strength within the cytoplasmic ground substance must be restricted to formed structures that show a specific gravity different from that of the surrounding medium Thus the demonstration of the bodies displaced after ultracentrifugation with intact morphology is strong evidence for their actual existence in the living cell In a nutshell objects which don t exist cannot be displaced!

Figure 91 shows the result of centrifuging liver cells submerged in Tyrode s solution for 30 minutes at 60 000 x gravity and photographed in ultraviolet light at 257 mμ in the unstained section The absorbing structures in the cytoplasm are clearly seen moved in the direction of the centrifugal force with their morphology preserved

Tullis How were you able to fix the cells in relation to the direction of centrifugation?

Lagerstedt In several ways usually in the tube

Tullis Attached or oriented to the side of it?

Lagerstedt Sometimes we fixed them in the tubes A small piece of tissue is placed in a centrifuge tube and after the centrifugation there is the piece of tissue in the bottom of the tube and then the fluid can be replaced We have used Tyrode s solution as suspension medium

Smetana Fresh tissue or fixed?

Lagerstedt A piece of liver in Tyrode s solution will sink to the bottom of the tube After centrifugation the piece is fixed em

Actually it appears that the basophilia is by no means a straight forward measure of the amount of nucleic acid present We have found in human biopsy livers instances in which basophilia was suppressed to the point where no conspicuous color was developed though the ultraviolet study showed that nucleic acid was actually present in some quantity (24)

Dr Popper I believe has found instances where the basophilia did not reflect a considerable change in nucleic acid content

Popper In experimental animals we found conditions in which the basophilia was markedly reduced but the nucleic acid content normal As reported here two years ago (17) Dr Szanto (25) had no difficulty in demonstrating cytoplasmic basophilia of the liver cells in biopsy specimen

Pollister There are many other angles to this biopsy material one being the curious fact that nucleic acid stains with fairly uniform success in autopsy livers a staining that is nuclease susceptible Tentatively we think that perhaps the protein nucleate bond is very firm in the fresh tissue hence not broken by the fixation and that by contrast it becomes somewhat looser during the period before autopsy

Hanger Do you suppose if autolyzed liver was used as a source of enzyme that reaction might be activated?

Pollister Dr Post has done a number of things like that so far with negative results

I should like to point out again that one is on very shaky ground if he concludes from a negative basophilia that nucleic acid concentration is necessarily low If possible this result should be checked by ultraviolet absorption or even better by direct chemical determinations on a mass of tissue

Smetana Were the biopsies fixed the same way?

Pollister Mostly acetic alcohol sometimes formalin

Lagerstedt One of the most important questions to be answered is whether the basophilic structures seen in the preparations also are present in that form and with that localization in the living cell The theory on the interaction between the nucleus and the cytoplasm in connection with the protein metabolism is largely based upon the localization of the ribonucleic acid containing cytoplasmic inclusions in close contact with the nuclear membrane during certain stages of their development

We tried to tackle the problem with two different experimental methods The first one consisted in use of the freezing drying methods the tissue is frozen in liquid nitrogen and afterwards

dehydrated at low temperature In that way changes in the localiza tion caused by the penetration of the tissue by fixatives is avoided as are the chemical changes accompanying the fixation in fluids

The other method consisted of submitting pieces of fresh tissue to ultracentrifugation The theory is that if cell constituents can be moved within the cells in the centrifugal field with unchanged morphology and if this morphology is always found using different fixatives that is good evidence that the structures are also present with those morphologic features in the living cell

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TABLE XX

Distribution of the Ultracentrifuged Material

No of Animals	No of *Centrifuged* Liver Pieces	Centrifugal *Force* g = Gravity	Centrifuging Time at *Maximal* Centrifugal Force in Min
5	10	40 000×g	10
2	6	60 000×g	10
2	6	—	20
2	6	—	30
2	6	—	40
6	12	120 000×g	10
4	8	—	20
4	8	—	30

character of the cytoplasmic structures after staining with gallocyanin chrome alum shows that the greater part of the ribonucleic acid of the cytoplasm is concentrated in the cytoplasmic inclusions The remaining parts of the cytoplasm absorb the ultraviolet light but faintly and show only inconspicuous basophilic properties

Thus there appeared to be a distinct discrepancy between the cytological findings and the structures isolated by Hogeboom Schneider and Pallade While in the intact cell the basophilic cytoplasm inclusions are well defined voluminous formations the fraction more rich in ribonucleic acid is found to consist of submicroscopic particles In the present studies an attempt has been made to bridge this discrepancy through examination of intact liver cells in ultraviolet light after ultracentrifuging at different rates and after various lengths of time The main interest here is attached to changes in the location and form of the cytoplasmic basophilic inclusions in relation to corresponding changes within the mitochondrial fraction

It is obvious that for comparative studies on basophilic cytoplasm inclusions and mitochondria under these experimental conditions examination of both structural elements in the same specimen offers the best possibility for reliable observations Both basophilic cytoplasm inclusions and the mitochondria can be presented simultaneously *in frozen-dried specimens* But in the preparation of the



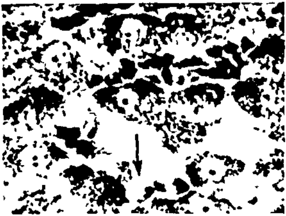
FIGURE 91 Pl... microns Car... gravity The nucleoli and ... s ons are moved in the direction of the centrifugal force their morphology is preserved (x1000)

bedded and sectioned then we are able to study the sections in the ultraviolet microscope or after staining the mitochondria as a control some pieces remain in Tyrode's solution for 60 minutes without centrifugation

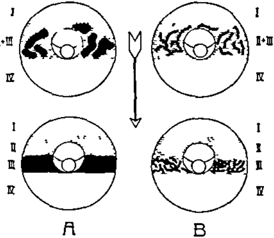
The results reached through these investigations and the different experiments performed are evident from Table XX and Figures 92A and B 93 94

For the present studies it is of particular interest that it has been possible to isolate a number of nucleic acid containing fractions from the cytoplasm of liver cells Of these fractions only one the mitochondrial fraction could be associated directly with well known structures in the cell Hogeboom Schneider and Pallade (26) have apparently been able by analysis of identified mitochondria to show that these make up about 19 per cent of the total ribonucleic acid content of the liver These authors however found the greater ribonucleic acid fraction (50 per cent of the total ribonucleic acid content of the liver) to be localized to submicroscopic particles of 100 mμ in size

A study of the pictures of liver cells in ultraviolet light at 257 mμ here reproduced as well as the pictures presenting the basophile



...ntrifuged for 20 ...ntrifugal direction ...ut have preserved ...empty zones are



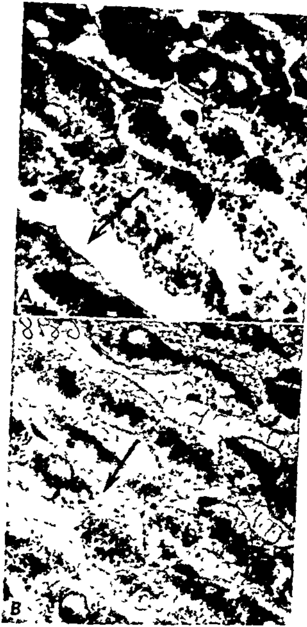


FIGURE 92 Sections from pieces of normal liver centrifuged at 60 000 x gravity for 30 minutes 5 microns The arrow indicates the centrifugal direction (x1000)

A Mitochondria stained cells The mitochondria are lying in a weakly staining homogeneous ground substance The centrifugal and centripetal optically empty layers are clearly visible

B Carnoy fixation Photomicrograph of unstained section at 257 millimicrons Strongly absorbing masses make up a layer containing the nucleus also The cytoplasm inclusions can no longer be made out as morphologic entities A centrifugal optically empty zone is conspicuous A finely granular slightly absorbing cytoplasm is seen centripetally Cf the scheme in Figure 91

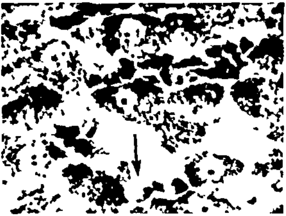
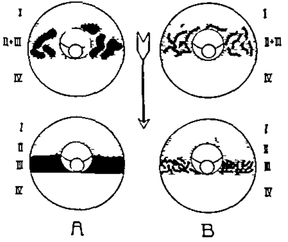


FIGURE 93. Mitochondria stained section from normal liver centrifuged for [illegible] minutes at 120 000 x gravity. 5 microns. The arrow indicates the centrifugal direction. The mitochondria have accumulated at the level of the nuclei but have preserved their individual morphology. Centrifugal and centripetal optically empty zones are seen (x1000)



present material it was not practicable to employ the freezing drying method Therefore it was decided to employ the Carnoy fixation for examination of basophilic cytoplasm inclusions and the Regaud fixation for the study of the mitochondria

The animals were killed by decapitation and small cubic pieces of liver (about 4 mm wide) were removed as quickly as possible Some pieces from each liver were placed directly in the fixation fluid others in Tyrode s solution There they were left as long as the experiment lasted Finally some pieces were placed in centrifuging tubes containing Tyrode s solution at a temperature of + 15

For these experiments an air driven ultracentrifuge of the Beams type (27) was employed rotating in vacuo with an effective radius of 88 millimeters After the centrifuging no increase in temperature could be ascertained in the Tyrode s solution on control measurements Table XX gives the centrifuging times at different rates It is to be pointed out that the recorded centrifuging times refer to the time after the maximal rate was obtained

After centrifuging the small blocks of tissue were fixed in Carnoy s or Regaud s fixation fluid together with the control blocks which had been left standing in Tyrode s solution for the same length of time The paraffin blocks were cut parallel with the centrifugal direction All the sections were 5 μ in thickness The sections were investigated in the ultraviolet microscope at 257 $m\mu$ after staining according to Regaud

As is evident from the findings when basophilic cytoplasm inclusions are submitted to centrifuging sufficiently long or at a sufficiently high rate of revolution they lose their individual morphology and enter into the formation of a strongly absorbent equatorial layer (Figure 92B) Under corresponding experimental conditions the mitochondria may be located in the same zone with preservation of their morphologic features (Figures 92A 93) Thus in both experimental series we meet with the formation of the four characteristic layers Apparently there can be no doubt that basophilic cytoplasm inclusions and the mitochondria become localized to the same zone (Figure 94)

In the Carnoy fixed or frozen dried specimens the mitochondria cannot be distinguished with certainty by photography in ultra violet light It has not been possible in the specimens examined to verify with certainty some findings reported in the literature about mitochondrial structures in the form of absorbent marginal structures surrounding an optically empty zone (28) After the mitochondria have gathered into more compact equatorial layers through

centrifuging, the possibility naturally arises that they may bring about, in part, the absorption observed But this does not alter the observation that the basophilic cytoplasm inclusions the most absorbent constituents of the cytoplasm are responsible for the major part of this absorption, as they can be followed during their aggregation into this layer

The possibility of basophilic cytoplasm inclusions being built up of aggregates of mitochondria, which as a result of the centrifugation break into pieces and enter the equatorial layer as mitochondria, is cancelled, however, by chemical as well as morphologic findings Hogeboom, Schneider, and Pallade (26) were able to isolate the entire mitochondrial fraction even by means of such a relatively slight centrifugal force as 24 000 x gravity for 20 minutes The supernatant fraction was found not to contain mitochondria but merely the submicroscopic particles rich in ribonucleic acids These particles are not likely to have been broken-up mitochondria, as the mitochondria in the intact cell are able to stand centrifugation for 30 minutes at 120,000 x gravity without undergoing any morphologic changes

It is reasonable to assume that the structures in the intact cell are more resistant to physical forces than when suspended in a cell foreign medium But in the experiments reported by the above-mentioned investigators, the isolated mitochondria were invariably of normal morphology and no broken mitochondria could be seen in the preparations

It is obvious, then, that a preponderant part of the ribonucleic acid content of the liver cell cytoplasm is localized to the sub microscopic particles, whereas the greater part of the ribonucleic acid content of the intact cell is found in basophilic cytoplasm inclusions On centrifugation the basophilic cytoplasm inclusions accumulate in the same layer as the mitochondria, and after centrifugation at a high rate for a considerable length of time, the baso philic cytoplasm inclusions in this layer undergo homogenization into a fine, granular substance Considering these factors, then, the only possible explanation appears to be that basophilic cytoplasm inclusions give rise to the submicroscopic particles in the isolated fractions

If we hypothetically assume that the basophilic cytoplasm inclusions are built up by the ribonucleic protein rich microsomes, will this throw any light on the question of the mechanism involved in the production of the cytoplasmic ribonucleic proteins?

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Lagerstedt I don't remember that Hydén made any general conclusions about that

Popper It is interesting in this respect that in poliomyelitis the first morphologically recognizable lesion is an alteration or disturbance of the RNA in the ganglion cells (the so called Nissl granules) This appears before any other lesion (32) and supports quite well what just has been said

Lagerstedt I think so That is why I asked Dr Mirick if you had made any studies in your material with cytochemical methods

Mirick Your point is a good one I am sorry I have no answers I am stimulated to try to get some

Hanger Have you made studies on hepatitis?

Lagerstedt Not from this point of view

Watson Dr Bang working with the electron microscope hasn't yet seen anything that looks like virus particles as they are ordinarily recognized

Shorr Have any respiratory measurements been made on liver cells that have been centrifuged and segmented in this fashion to determine whether they retain their capacity for respiration?

Lagerstedt Measurements have been made on the respiratory capacity of sea urchin eggs submitted to centrifugation* I don't think they have been made in the liver

Shorr I wonder how large a portion of liver it would be practical to treat by centrifugation?

Lagerstedt There is one objection if you are interested in the cytology It will be hard to fix the specimen satisfactorily if it is too large

Shorr I was thinking of removing them and studying certain metabolic reactions

Lagerstedt There is one old story not too pleasant to remember That is the time when an egg from Ascaris suum was centrifuged for about 900 000 x gravity I think for 10 hours After that treatment no structures within the cell membrane could be seen at all only a delicate nuclear membrane but all other constituents were homogenous and finely granulated However that egg was quite easily fertilized and there appeared a nice little worm in due time

Shorr One would have to consider the subsequent effects on the cells of the initial period of hypoxia

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* Investigations by Chantrenne (33) relate to this question He found a decrease in the oxygen uptake by 50 per cent through inactivation of the Warburg Keilin system S L

This question cannot be answered yet, but there are certain findings which indicate that at least the mitochondria seem to be able to duplicate within the cytoplasm Gustafson (29) showed in the sea urchin's blastula and gastrula that the number of mitochondria could change considerably under different conditions It is also well known that eggs can be centrifuged during cleavage and in that way one cell with mitochondria and the other without visible mitochondria obtained After some time, mitochondria appear again These observations have led to the hypothesis that mitochondria could arise from some precursor, possibly microsomes an old concept, indeed, already suggested by Meves (30)

Several attempts have been made to identify the microsomes with plasmagenes as well as viroidal bodies found in the cytoplasm and containing nucleic acids It is tempting to assume that changes in the amounts of nucleic acids and proteins in the cytoplasm might be brought about through changing the number of microsomal and mitochondrial bodies in the cytoplasm This seems to be a most interesting working hypothesis, but the type of work can only be performed within several, technically different fields of research

Smetana Dr Lagerstedt talked about viruses and their possible relation to structures in the cells Inclusion bodies in some of the cells are seen in virus diseases Is there any relationship between the inclusion bodies in nuclei and the nucleolar mass which you have shown?

Lagerstedt The main investigation with cytochemical methods for localizing viruses in cells has been done by Hydén (31) He investigated infected cells in the ultraviolet microscope and made measurements of the amounts of nucleic acid present at different times after infection I don t think he ever stated what connection he found between the accumulations of virus and the inclusion bodies I have no remembrance of that

Smetana I understood you to say the contact between the virus and the cell occurs through these bodies rather than with any thing else

Lagerstedt In certain virus diseases for instance, in Molluscum contagiosum the virus is seen accumulating close to the nuclear membrane Thus, under those conditions there is morphologically a close connection between this part of the protein forming system and the viruses and I reported that probably the virus will destroy some part of this protein forming system

Popper Were there any differences in the various viruses depending upon whether the virus consisted of DNA or RNA?

Lagerstedt I don't remember that Hydén made any general conclusions about that

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Popper I should like to answer the question Dr Hanger raised about hepatitis based on histologic observation of the cytoplasmic basophilia and not on ultraviolet absorption study or chemical analysis As mentioned two years ago (17), viral hepatitis presents in pyronin stains, a characteristic picture in contrast to the chemical type of hepatitis To us, the alterations of the cytoplasmic baso philia is one of the most diagnostic of morphologic changes In viral hepatitis, isolated cells lose the basophilia that is, one cell loses it and may subsequently become necrotic, whereas a neigh boring cell retains its normal basophilia In early stages, the loss of the basophilic granules from individual cells contrasts markedly to the findings in the toxic hepatitis in humans or animals (e g, due to carbon tetrachloride) which show a diffuse loss of the basophilia in the entire centrolobular zone This is in agreement with the findings of Dr Mallory Dr Smetana and his co workers (34) have described, in non fatal viral hepatitis necrosis of isolated liver cells

I should like to pose two questions dealing with problems which came up in the Ninth Liver Injury Conference One deals with the appearance of the nucleoli In cirrhosis, the appearance of liver cells varies considerably, as one would expect There are groups of cells which are rich in cytoplasmic basophilic material, in others the cytoplasm appears empty and practically free of basophilic material. In the latter type of cells Szanto (25) usually found very large nucleoli similar to what Stowell (35) saw after prolonged protein free diet A possible explanation for the large nucleoli and absence of cytoplasmic basophilia may be an association of in creased formation of ribonucleic acids with increased turnover or destruction of protein I wonder whether a better explanation is available today

Brachet (36) and Caspersson (37) have assumed and their whole work is based on this assumption that *ribonucleic* acid plays an important rol [illegible] and represents supposedly, the organelle for [illegible] convince myself from the stu[illegible]uld be accepted without doubt *The findings presented today strongly support* it I wonder whether Dr Best would permit a discussion of this basic problem

Best Can that be answered briefly, Dr Lagerstedt?

Lagerstedt It could be discussed but not definitely answered We have as quite a constant finding that in connection with protein formation there is an increase *in the amount of ribonucleic acids*

There have been some investigators who have suggested that the nucleic acids, which easily precipitate proteins might facilitate protein syntheses by removing the reaction products of the synthesis

Dr Stetten is interested I think, in some studies on nucleic acid Have you ever considered these possibilities from the purely chemical point of view? Is it possible that a chemist could give some clues?

Stetten Nothing immediately obvious, I think There are relatively few simple systems which would appear to make peptide bonds, so far as I am aware Those systems which have been studied in the isolated state which synthesize peptide bonds as in hippuric acid or glutathione, have not been shown to require nucleic acids

Hartroft I should like some information about the techniques which you have been employing Dr Pollister Do you use a quartz microscope or a reflecting microscope? In measuring the photometric absorption how do you control errors that might arise from variations in the thickness of the section or the intensity with which it is stained?

Pollister We have been using both the quartz monochromatic objectives and the reflecting types with about the same result

Hartroft What type of reflecting microscope do you employ?

Pollister We have both the Bausch and Lomb and American Optical Company types of reflecting objectives

Hartroft Does not the central stop in those employing spherical mirrors interfere with resolution when the substage aperture is reduced to eliminate glare?

Pollister It is astonishing that the resolution is so very good in the visible light I have never seen a refracting objective of comparable numerical aperture which would give, at a magnification of 800 or 900, anywhere near the quality of the image obtained with the reflectors And, of course, there is no chromatic aberration, especially in the totally reflecting AO type

Now, as to the other question there are errors of all sorts, and I would be glad to discuss them at length

Best I think it should be done privately

Pollister I might merely say what we think we can do We look for relative changes, we do not think we can determine absolute amounts, but the relative amounts are extremely useful in a great deal of biological material For a fuller discussion I refer you to a recent paper (38)

Schwartz Dr Grafflin this morning introduced the technique of fluorescence microscopy with special reference to its use in the study of excretion of fluorescent dyes by the liver The studies of

fluorescence microscopy which I should like to consider very briefly are presented not because they have already been applied to the study of the physiology, biochemistry, or anatomy of the liver but because the technique seems to have some promise of being of value in future studies in these fields

The goal set for us in the work was a twofold one Originally, we wanted to study the fluorescence of different tissues by fluorescence microscopy and to determine by visual or photographic means *the characteristic changes in the fluorescence pattern* in various diseases Such studies have been made by a number of investigators in the case of liver tissue, principally by Dr Popper Secondly, we wanted to extend this work to a more quantitative definition of the fluorescence spectra by means of photography or other techniques

Two methods have been employed The first involves photography of the fluorescence spectrum and subsequent analysis of the spectrum on a self recording densitometer This method has certain disadvantages It requires long exposure at low levels of light intensity, and under such conditions the grain darkening in the film may be so great that it is difficult to evaluate the fluorescence spectra obtained unless the fluorescence is very intense In general one half hour or longer exposures are made Such long exposures may destroy some of the fluorescing compounds We have therefore built a self recording microspectrophotometer, as shown in Figure 95

Seen here is the apochromatic microscope employed and just beyond it a high intensity AH6 mercury lamp cooled by a recirculating water system Filtered light from this lamp enters the microscope On the microscope stage is placed the tissue section or solution to be studied In the eyepiece of the microscope is a microspectroscope built by Steinheil and Sons and brought back from Germany by Dr Watson three years ago This unit has a number of special features

1) A lower eyepiece which permits direct visualization of the microscope field
2) An upper eyepiece which permits visualization of a slit whose size and position can be varied by two diaphragm controls The slit can then be closed around the cell or other areas to be analyzed
3) The same eyepiece is used as a spectroscope by rotating a dispersion prism into position in the adjacent drum The position of fluorescence or absorption bands can be directly measured on a calibrated drum scale The cross hairs in the

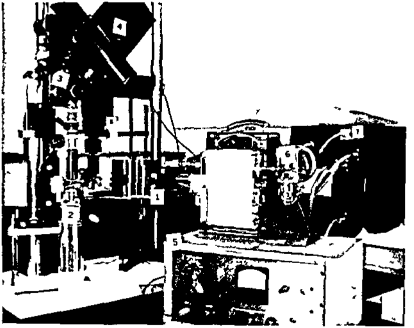


Figure 95 Self recording microspectrofluorophotometer and auxiliary units
1 Lamp housing
2 Cooling unit for tissue or solution (the microscope [not numbered] employs vertical illumination when used with this cooling unit)
3 Microspectroscope
4 Phototube and housing
5 D C amplifier

and 579 milli

microns

spectroscope may be illuminated with appropriately filtered light when necessary in fluorescence studies

4) A camera extends upward to the right of the drum
5) Glass or quartz prisms in the drum are used to disperse visible or ultraviolet light to focus the spectrum at the camera exit These spectra may be photographed and analyzed with a recording densitometer
6) Because of the deficiencies of photography as noted earlier the spectra are now analyzed directly by a system which employs a phototube which scans the spectrum an amplifier, and an Esterline Angus Recorder

Variable slits are interposed between the camera exit and the phototube to *transmit highly monochromatic light* (*1 mμ band* width or less) The phototube housing and slit are coupled through a gear system and a selsyn drive unit to the Esterline Angus Recorder The position of the slit or wave length *can be read from* a calibrated drum scale on the driving mechanism Light impinging on the phototube at any moment is amplified approximately 1 000 000 times and then fed into the amplifier where further amplification over a 100 000 fold range may be produced The current fed into the recorder thus gives a continuous plot of light intensities at successive wave lengths or of the fluorescence spectrum A complete spectrum may be produced in from approximately one half to three or four minutes

The phototube is cooled with dry ice to reduce its so called dark *current We have also employed a liquid nitrogen cooling system* to cool tissue sections to increase stability of fluorescent compounds

The work to date has been limited almost entirely to the development of the apparatus to the elucidation of various factors involved in achieving reproducibility a high degree of resolution etc

Figure 96 illustrates the plot obtained of the mercury doublet at about 577 and 579 millimicrons Almost complete separation of these two peaks will be noted As indicated by this curve a resolution down to a few Angstrom units is possible Because we are especially interested in various porphyrin compounds whose fluorescent spectra may be only two or three millimicrons apart the need for a high degree of resolution is a basic one

We have studied the fluorescence in liver sections obtained from normal individuals and some patients with cirrhosis and other types of liver disease In general the hepatic cells in the unstained state exhibit a bluish green fluorescence In the cytoplasm there may be many small granules of orange or reddish fluorescent material The bile duct contents may exhibit fairly intense red fluorescence One has the impression that there are not only differences in the shade of green fluorescence seen in the same liver but more especially there seem to be qualitative differences in fluorescence color observed in pathologic livers We therefore hoped that the fluorescence spectra might be abnormal in diseased livers and that these abnormalities might serve as clues to the further chemical analysis of these livers

To date we have prepared relatively few spectra as might be expected different types of spectra are observed In general one observes a very broad continuous spectrum in the green extending

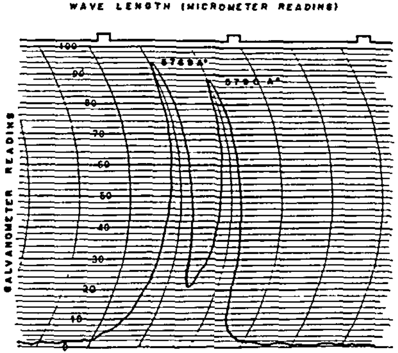


FIGURE 96 Resolution of the mercury doublet by the self recording microspectrofluorophotometer

into the yellow and somewhat into the red region of the spectrum Sometimes, one observes two peaks rather than a single broad peak Some spectra are much sharper than others We have no idea what these differences in fluorescence spectra mean Work is continuing in a further study of the fluorescence pattern of normal and abnormal livers to determine how consistent these differences may be and to see what biochemical significance may be attached to them

Grafflin Dr Watson and his colleagues are to be warmly congratulated for developing this equipment which should give them access to extraordinarily interesting material

Knisely You could begin to use this instrument for analysis of infinitesimal amounts of material for example, with paper chromatography could you not?

Schwartz Probably With the fluorescing substance on paper, however, there would be some difficulty in quantitative interpreta-

tion of fluorescence intensity What we have done as a rule is to concentrate the material take it up in small capillary pipettes which may have an inner diameter of from one tenth to one half a millimeter seal the ends of the capillary tube with paraffin to prevent evaporation and place it on the microscope stage The total volume used for analysis may if necessary be less than one thousandth of a milliliter

Knisely It is time to go back and start a lot of chemistry of *microsamples Recall the exquisite research on the quantitative* chemistry carried out on very small samples of fluid obtained from anatomically *defined* parts of the kidney nephron the work of Professor A N Richards and his school It began with Richards and Schmidt 1924 25 and continued with the assistance of Professors Wearn Hamilton Walker and others (39 40 41 42 43 44)

It is now possible to get samples from many anatomically defined almost microscopic parts of living animals The double ball joint *micromanipulator invented by Fritz Buchthal (45) makes the ob*taining of the sample possible The new instrument Dr Schwartz is describing makes the analyses possible It should now be possible *to carry out analyses of functions of specific parts of organs such* as the liver

Schwartz One big advantage of fluorescence microscopy over *absorption microscopy is the fact that in the latter instance sensi*tivity is limited by the concentration of the material one has to deal with Many of these compounds must be present in a concentration of from one to four or five per cent depending upon the specific absorption of the compound The use of amplifying systems is of no value here whereas in the case of fluorescence one is able to achieve an extreme degree of sensitivity by increasing the amplification in various parts of the system

Popper The rapid fading of the originally very bright fluorescence is rather characteristic for vitamin A and permits its localization in tissue sections (46) After the fading of the vitamin A fluorescence *other fluorescent details may become recognizable* such as a red fluorescence in the liver which may persist for considerable time half an hour or longer Also other fluorescent details especially pigments may become apparent

Schwartz For that reason we had hoped one might stabilize the fluorescence by building a cooling unit to cool the tissue to the temperature of liquid nitrogen We have such a system involving a copper flask and two Dewey flasks We can measure the temperature in various areas and can get pretty close to the temperature

of liquid nitrogen. With the cooling technique we find there is some instability; for instance, in the porphyrins there may be an increase in the duration of fluorescence by a factor of four or five, but it has not completely eliminated photo-oxidation. One other advantage of cooling is that in many instances the fluorescent spectra are considerably sharpened and much more detail is apparent in the highly cooled material than is visible at room temperature.

Knisely: Is there any chance that the rates of decay could be used for any type of interpretive work?

Schwartz: We hope it will be the case. It is a feature of this Recorder that the speed of the plotting can be varied considerably. It is also possible to change the wave length scale by altering gears in the driving mechanism. We have reason to believe that some porphyrins are less stable than others. We plan to study this possibility by setting the camera slit at about 620 mμ and quickly scanning the spectrum from 620 to 635 millimicrons. The gears are then reversed and the spectrum scanned from 635 to 620 mμ and so on back and forth every minute or so. Differences in stability should be revealed by progressive changes in the spectrum obtained.

Popper: I have been interested in fluorescence microscopy for many years, using it in a somewhat primitive way. I see now that with the arrangement just presented a dream has come true, and I am confident that a large number of important observations are going to be made with this outfit. Congratulations. I have just one question. Does a very strong vitamin A fluorescence in the first few seconds of exposure to ultraviolet light not disturb your observation until the vitamin A fluorescence has somewhat decayed? When the slide goes under the microscope you should get an overwhelming fluorescence in the normal liver.

Schwartz: It hasn't disturbed us, but only because we haven't studied the matter. It will become a factor when we get around to plotting spectra of freshly exposed sections.*

Popper: We had some difficulty with the problem of fixation in fluorescence microscopy. If tissues are kept in formalin for several days, fat originally not fluorescent acquires a secondary fluorescence. On the other hand, in fresh frozen sections certain types of fluorescence interfere with the recognition of other types. However, I am sure these are not insurmountable difficulties. Scandinavian studies (47) concerning fluorescence microscopy of tissue embedded by

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* As noted earlier, it is hoped that increased stability may be achieved by cooling the section down to liquid nitrogen temperature.

drying freezing techniques, especially in localizing sulfonamides, appear to me most promising

Smetana Are fluorochromes used in connection with this instrument?

Schwartz Yes, indeed Many of the ordinary stains are actually fluorochromes but, as I say, the work up to now has been almost entirely on the development of the apparatus Applications have come much more slowly than we would have liked, but we felt that the important thing was to get an apparatus which is as good as we want it to be Applications will come rapidly enough when the apparatus is finally perfected

I might mention some preliminary studies with Dr Lane Williams Rats were treated with varying amounts of carbon tetrachloride and then injected with Rose Bengal They were sacrificed at various intervals and the sections studied both by ordinary microscopy and by fluorescence microscopy It was found that the fluorescence analysis was a more sensitive test for the presence of Rose Bengal in the hepatic cells, since increased yellowish fluorescence was observed in sections which appeared normal with ordinary technique

Hartroft How do you visualize your field directly?

Schwartz Actually, a considerable amount of detail can be seen in the unstained liver The nuclei, cytoplasm, particles, etc, show up well

Watson Some of the most beautiful preparations that Dr Schwartz and Dr Schmid have got have been of bone marrow normoblasts and they are full of porphyrin, most of the porphyrin initially is in the nucleus The evidence strongly suggests that porphyrin formation begins in the nucleus rather than the cytoplasm, which was rather surprising to us, but one can identify cells on the slide, then take the slide out and stain it with an ordinary technique and go back and look at the cells and see exactly which ones fluorescence was identified in For example, in certain cases [illegible]

are two colonies of cells [illegible]

do They can be looked at afterwards stained with Wright's stain and they look identical and one without porphyrin fluorescence cannot be distinguished from another right next to it with a large amount We don't understand this interesting phenomenon

Best I think we shall have to draw to a close Dr Lagerstedt, to bring our session to a conclusion is there anything you would like to say?

Lagerstedt I have certainly said enough in one day I only hope that these remarks of mine will make it as clear to you as it is to me that in the field of liver cytochemistry and histochemistry, there are very good possibilities for thoroughly fruitful cooperation between cytologists, biochemists, and people working with yet other methods I hope that the conclusions which can be drawn from a study of the properties of isolated particles within the liver cell will aid in having this work start as soon as possible I am convinced that there is much information to be gained about the internal metabolism in general through studies on this type of cell

Best That is a good point at which to end the formal side of this Conference

REFERENCES

1 BERG W Über den mikroskopischen Nachweis der Eiweiss speicherung in der Leber von grosseren einheimischen Haus tieren und frei lebenden sudamerikanischen Tieren *Ztschr f mikr anat Forsch* 30, *38 (1932)*

2 EINARSON L A method for progressive selective staining of Nissl and nuclear substance in nerve cells *Am J Path* 8, *295 (1932)*

3 LAGERSTEDT S Gallocyanin staining as a specific method for production of protein inclusions in liver cells *Acta anat* 2, *392 (1947)*

4 ——— The quantitative estimation of basophilia through gallocyanin-chromalum staining *Acta anat* 5, *217 (1948)*

5 STENRAM U The specificity of the gallocyanin-chrome alum stain for nucleic acids as studied by ribonuclease technique *Exper Cell Res* (In press)

6 HYDÉN H Protein metabolism in the nerve cell during growth and function *Acta physiol Scandinav Suppl 17 6, (1943)*

7 CASPERSSON T Über den chemischen Aufbau der Strukturen des Zellkernes *Skandinav Arch f Physiol Suppl 8* 73, *(1936)*

8 LAGERSTEDT S Investigations on the proteinaceous inclusions of the liver cell cytoplasm in ultraviolet light *Acta anat* 3, *81 (1947)*

9 COLE, P A and BRACKETT F S Technical requirements in the determination of absorption spectra by the ultraviolet micro scope *Rev Scient Instruments* 11 *419 (1940)*

10 LAGERSTEDT S Effect of microincineration on the proteinaceous *inclusions in normal rat liver cytoplasm Acta anat 3, 190 (1947)*

11 ——— Ultraviolet spectrum of the proteinaceous inclusions in normal rat liver cytoplasm *Acta anat* 3, *265 (1947)*

12 ——— Cytochemical investigations on the protein metabolism of liver cells *Exper Cell Res Suppl* 1, *380 (1947)*

13 ——— Cytological studies on the protein metabolism of the liver in rat *Acta anat Suppl* 9 1 *1 (1949)*

14 ——— Den morfologiska bilden av nukleinsyrehaltiga aggvitor i levercellcytoplasman *Nord med tidskr* 32, 2662 (1946)

15 STOWELL, R E Nucleic acids and cytologic changes in regenerating rat liver *Arch Path* 46, 164 (1948)

16 ——— Alterations in nucleic acids during hepatoma formation in rats fed p dimethylaminoazobenzene *Cancer* 2, 121 (1949)

17 POPPER, H Correlation of hepatic function and structure based on liver biopsy studies *Liver Injury* Hoffbauer, F W, Editor Trans Ninth Conf New York, Josiah Macy, Jr Foundation, 1950 (p 9)

18 FARBER, E, *et al* Correlation between cytoplasmic basophilia and the nucleic acid content of the liver *Arch Path* 51, 399 (1951)

19 KREMER, J Die morphologische Gestaltung der Gallensekretion *Ztschr f mikr anat Forsch* 33, 485 (1933)

20 GRAFFLIN, A L A histological study of fat distribution in guinea pig livers of chemically known fat content. *Anat Rec* 77, 473 (1940)

21 POLLISTER, A W Quelques methodes de cytologie chimique quantitative *Rev d hemat* 5, 527 (1950)

22 OSTER, G, and GRIMSON, H The interaction of tobacco mosaic virus and of its degradation products with dyes *Arch Biochem* 24, 119 (1949)

23 DOUNCE, A L Physiology of the cell nucleus A symposium *Exper Cell Res*, *Suppl* (In press)

24 POLLISTER, A W, *et al* Resistance of ribonucleic acid to basic staining and ribonuclease digestion in the human liver *J Nat Canc Inst* 12, 242 (1951)

25 SZANTO, P B, and POPPER, H Basophilic cytoplasmic material (pentose nucleic acid) distribution in normal and abnormal liver *Arch Path* 51, 409 (1951)

26 HOGEBOOM, G H, SCHNEIDER, W C, and PALLADE, G E Cytochemical studies of mammalian tissues, isolation of intact mitochondria from rat liver, some biochemical properties of mitochondria and submicroscopic particulate material *J Biol Chem* 172, 619 (1948)

27 BEAMS J W An apparatus for obtaining high speeds for rotation *Rev Scient Instruments* 1, 667 (1930)

28 OPIE, E L Mobilization of basophile substance (ribonucleic acid) in the cytoplasm of liver cells with the production of tumors by butter yellow *J Exper Med* 84, 91 (1946)

29 GUSTAFSON, T, and LENICQUE, P Studies on mitochondria in the developing sea urchin egg *Exper Cell Res* 3, 251 (1952)

30 MEVES F Cf Hertwig, G Das Cytoplasm A Die Definition des Cytoplasmas und die Hypothesen uber seinen morphologische Bau *Hdb d mikr Anat d Menschen* I/1, 212 (1929)

31 HYDEN H The nucleoproteins in virus reproduction *Cold Spring Harbor Symp Quant Biol* 12, 104 (1947)

32 BODIAN D Histopathologic basis of clinical findings in poliomyelitis *Am J Med* 6, 563 (1949)

33 CHANTRENNE H Recherches sur des part cules cytoplasm que de d mens ons macromolecula res r ches en ac de pentosen nucléique *Enzym ologia* 11 *213 (1943)*
34 KELLER T C GIGES B and SMETANA H F H stopatholog study of acute nonfatal hepat t s *Mil S rgeo* 109 *425 (195)*
35 STOWELL R E Alterat ons in nucle c acids dur ng hepaton format on in rats fed *p* d methylam noazobenzene *Ca cer* 2 *121 (1949)*
36 BRACHET J *Cl e cal Embryology* New Yo k Inters ence Publ shers 1950
37 CASPERSSON T *Cell Growth a d Cell Fu ctio A C clem cal Study* New York Norton 1950
38 POLLISTER A W M cro pectrophotometry of fixed ells by v s ble l ght *Lab I vest* 1 *231 (1952)*
39 RICHARDS A N and SCHMIDT C F A descr pt on of th glomerular circulat on in the frog s k dney and observat on concern ng the act on of adrenal n and var ous other substan upon it *A J Phys ol* 71 *178 (1924)*
40 WEARN J T and RICHARDS A N Observat ons on the o pos t on of the glomerular urine w th part cular referen c the problem of reabsorpt on in the renal tubules *A J Pl* 71 *209 (1924)*
41 RICHARDS A N Ur ne format on n the amph b an k dn *Harvey Lect* 30 *93 (1934 35)*
42 ——— Croonian lecture processes of urine format on *P Roy Soc Lond s B* 126 *398 (1938)*
43 ——— Phys ology of the kidney Wesley M Carpen er L *ture B ll Ne York A ad Med* 14 *5 (1938)*
44 WALKER A M *et al* The collect on and analys s of fl from s ngle nephrons of the mammalian kidney *A J Pl* 134 *580 (1941)*
45 BUCHTHAL F E n neuer M kroman pulator m t Zusatzgera (M kromesser und unpolar v erbare M kroelektroden) *Z / u se cb M kr* 58 *126 (1942)*
46 POPPER H D str but on of vitam n A n t ssue as v ual z by fluorescence m croscopy *Phy iol Rev* 24 *205 (1944)*
47 HELANDER S On the concentrat ons of some sulfan la derivatives n d fferent organs and t ssue structures a h pharmacolog cal study by means of fluorescence m croscopy w some aspects on poss b l t es of obta n ng h gh con entrat on chemotherapeut cs n certa n t ssues *A ta phys ol S d Suppl* 29 *10 1 (1945)*